Doctoral Dissertation

Identification, characterization, and regulation of the proteolytic system that degrades uncomplexed SecY subunit of protein translocase in the *Escherichia coli* plasma membrane

Akio Kihara

Department of Cell Biology, Institute for Virus Research, Kyoto University

Abstract

SecY is an integral membrane protein which forms a hetero-trimeric complex with SecE and SecG and functions as a subuint of protein translocase in *Escherichia coli*. SecY can exist stably in the cell only when it is in association with SecE. Thus, uncomplexed forms of SecY, which are produced when *secY* is overexpressed over *secE* or when *secE* is underexpressed, are eliminated by proteolytic degradation. To identify the proteolytic system responsible for the degradation of uncomplexed SecY, we isolated mutants in which oversynthesized SecY was stabilized. We found that loss-of-function mutations of the *ftsH* (*hflB*) gene stabilized oversynthesized SecY. Overproduction of the wild-type FtsH protein accelerated the degradation of SecY. These results indicate that FtsH is required for degradatin of SecY *in vivo*. Purified FtsH protein catalyzed ATP-dependent proteolysis of SecY *in vitro*, thus establishing that this membrane-bound ATPase acts as a protease to eliminate unassembled SecY subunit from the membrane. It was found that overproduction of SecY in the *ftsH* mutant leads to an inhibition of cell growth and protein export. Thus, FtsH serves as a quality control machine to keep the integrity of the membrane by avoiding accumulation of unwanted membrane proteins.

We also isolated SecY-stabilizing and partially dominant mutaions in the *hflK*, *hflC*, and *yccA* genes, all of which encode membrane proteins. HflK and HflC form a complex (HflKC) in the cytoplasmic membrane and has been believed to be a protease that degrades λ CII protein, required for lysogenization of this bacteriophage. Although FtsH-His_6-Myc ATP-dependently degraded CII, our results all argued against the prevailing view that HflKC is a protease. The *ΔhflK-hflC* null mutaion did not stabilize SecY; it rather destabilized the SecY24 protein. The same mutation stabilized CII only under limited *in vivo* conditions. Even overproduction of HflKC stabilized CII and increased the λ lysogenization frequency. Furthermore, the proposed serine protease active site motif of HflC was dispensable with respect to the lysogenization controlling function. The HflKC proteins were found to have

their main extramembrane domains exposed to the periplasm, making it almost impossible for HflKC to degrade the cytosolic CII protein.

We demonstrated, by crosslinking, co-immunoprecipitation, affinity isolation using a histidine-tagged FtsH, and gel filtration experiments, that FtsH and HflKC form a complex. *In vitro*, HflKC lacked any detectable protease activity by itself, and it was rather inhibitory against the FtsH-catalyzed proteolysis of SecY or CII. These results, taken toghter, indicate that HflKC is not a protease. Most probably, it is a modulator of the FtsH function, and this modulation should be exerted across the membrane.

The mutation in *yccA* (*yccA11*) was found to affect an ORF (*yccA*) encoding a hydrophobic protein with putative seven transmembrane segments. The wild-type YccA protein was found to be degraded in an *ftsH*-dependent manner *in vivo*, but the YccA11 mutant protein, lacking 8 amino acid residues within the amino-terminal cytoplasmic domain, was refractory to the degradation. The YccA11 protein was found in association with the FtsH/HflKC complex. Thus, YccA11 seems to interfere with proteolysis by occupying a site in FtsH that is essential for recognition of other substrates. The lack of proteolysis of YccA11 seems to be due to the shortening of the cytoplasmic tail since reattachment of an unrelated sequence made it suspectible to FtsH again. The inhibitory action of YccA11 is somehow mediated by HflKC, since the deletion of *hflK-hflC* suppressed the *yccA11* phenotype. The *yccA11* mutation stabilized subunit *a* of the proton ATPase F_0 sector, another membrane substrate of FtsH, as well, but not the λ CII protein. These results suggest the existence of at least two recognition pathways for the FtsH-dependent protein degradation, only one of which is subject to the HflKC-dependent interference by the YccA11 mutant substrate. Finally, we demonstrated that HflKC can directly interact with YccA. We proposed that HflKC modulates the FtsH actions to different substrate proteins by interacting with membrane-embedded substrates of FtsH.

Abbreviations

SDS, sodium dodecyl sulfate

SDS-PAGE, SDS-polyacrylamide gel electrophoresis

DSP, 3, 3'-dithiobis-(succinimidyl propionate)

PMSF, phenylmethyl sulfonylfluoride

DTT, dithiothreitol

XG, 5-bromo-4-chloro-3-indolyl β-D-galactoside

tPEG, phenylethyl β-D-thiogalactopyranoside

IPTG, isopropyl β-D-thiogalactopyranoside

PCR, polymerase chain reaction

ORF, open reading frame

IS, insertion sequence

cAMP, adenosine 3', 5'-cyclic phosphate

Amp, ampicillin

Cm, chloramphenicol

TCA, trichloroacetic acid

CCCP, carbonyl cyanide m-chlorophenylhydrazone

Bla, β-lactamase

MBP, maltose binding protein

PhoA, alkaline phosphatase

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§ 1 Introduction

I. SecY and general background of this study

Several Sec proteins participate in the transport of secretory proteins across the plasma membrane of *E. coli*. These include SecA, SecB, SecD, SecE, SecF, SecG, and SecY (Ito, 1995; Wickner and Leonard, 1996; Duong *et al.*, 1997). SecB is a secretory protein-specific chaperone, which maintains presecretory proteins in translocation-competent conformation after their synthesis (Randall *et al.*, 1990). SecA, having ATPase activity, provides an energy for protein translocation together with the proton motive force (Lill *et al.*, 1989; Schiebel *et al.*, 1991). SecY, SecE, and SecG are principal integral membrane components of the translocation machinery (Ito, 1992; Nishiyama *et al.*, 1993), and they form a complex (Brundage *et al.*, 1990; Douville *et al.*, 1994; Douville *et al.*, 1995; Homma *et al.*, 1997) which is thought to provide an intramembrane pathway for preprotein transit. Reconstitution studies indicate that SecY and SecE can exhibit some basic translocatin activity in conjunction with SecA (Akimaru *et al.*, 1991). Thus, they are basic subunits of the translocation channel. SecD and SecF appear to play a role late in the translocation process (Matsuyama *et al.*, 1993; Duong and Wickner, 1997).

SecY and SecE span the plasma membrane 10 times and 3 times, respectively (Akiyama and Ito, 1987; Schatz *et al.*, 1989). SecY and SecE are synthesized roughly in an equimolar ratio in wild-type cells (Matsuyama *et al.*, 1992), and they immediately form a stable complex that does not dissociate measurably thereafter (Taura *et al.*, 1993; Joly *et al.*, 1994). Although SecY that is complexed with SecE is stable, uncomplexed form of SecY is highly unstable. For example, overproduced SecY is degraded with a half-life of about 2 min, while it is completely stabilized by the simultaneous overproduction of SecE (Matsuyama *et al.*, 1990; Taura *et al.*, 1993). SecY is also destabilized in the *secE501* mutant cells in which synthesis level of SecE is lowered (Taura *et al.*, 1993). Thus, SecE is the stabilizing partner of SecY. Genetic studies in our laboratory suggest that the fourth cytoplasmic domain of SecY is

important for its interaction with SecE (Baba *et al.*, 1994). A mutation in this domain, *secY24*, which alters Gly^{240} to Asp (Shiba *et al.*, 1984), has been shown to weaken SecY-SecE interaction and to cause degradation of the altered SecY24 protein at high temperature (Baba *et al.*, 1994).

The rapid elimination of uncomplexed forms of SecY may be important for cells to maintain the quality of the membrane. For instance, accumulation of potentially channel-forming SecY protein subunit may lead to uncontrolled collapse of chemical gradients in the absence of the partner SecE protein. Although several naturally or conditionally unstable proteins as well as the proteases responsible for their degradation have been studied in considerable detail in *Escherichia coli*, our knowledge about degradation of membrane proteins in this organism is still limited. The present work was originally aimed at identifying the proteolytic system responsible for the rapid degradation of SecY, and our results established that a membrane protein called FtsH is the protease acting on SecY. Further studies revealed that FtsH forms a complex with other membrane proteins, HflK and HflC, which most probably modulate activity and specificity of the FtsH protease.

II. ATP-dependent proteases in *Escherichia coli*

While the majority of *E. coli* proteins are stable, some are rapidly degraded *in vivo*. The unstable proteins include regulatory proteins whose functions are requiring during limited time ranges or under limited physiological conditions. In addition, abnormal proteins are often degraded rapidly to ensure integrity of celluler processes; they include misfolded proteins, mutant proteins, proteins encoded by foreign DNAs, and proteins that failed to make a complex with their partners. Although a number of proteases and peptidases are known in *E. coli* (Gottesman, 1996), those required ATP seem to be involved in important cellular functions. Five ATP-depndent proteases have been described in *E. coli*. They are Lon (Chung and Goldberg, 1981; Charette *et al.*, 1981), ClpAP (Hwang *et al.*, 1987; Katayama *et al.*, 1988), ClpXP (Wojtkowiak *et*

al., 1993; Gottesman *et al.*, 1993), HslUV (Yoo *et al.*, 1996; Rohrwild *et al.*, 1996), and FtsH (Tomoyasu *et al.*, 1993a; Tomoyasu *et al.*, 1995). The Clp proteases consist of ATPase subunits (ClpA and ClpX) which confer substrate specificity, and the proteolytic ClpP subunit (Tobias *et al.*, 1991; Gottesman *et al.*, 1993; Wojtkowiak *et al.*, 1993; Lehnherr and Yarmolinsky, 1995). HslUV is also a two-component enzyme consisting of a proteolytic (HslV) and an ATPase (HslU) subunit (Yoo *et al.*, 1996; Rohrwild *et al.*, 1996). Lon and ClpP are serine proteases (Amerik *et al.*, 1991; Maurizi *et al.*, 1990) and HslV is a threonine protease (Rohrwild *et al.*, 1996). The ATP-dependent proteases share some characteristic features.

First, like molecular chaperones, many of their components are induced by a heat shock. The *clpP*, *clpX*, and *lon* genes are located at 10 min contiguously on the *E. coli* chromosome, with *clpP* and *clpX* forming an operon and *lon* located downstream of *clpX* (Gottesman *et al.*, 1993). Their transcription is initiated by σ^{32}, the heat shock σ factor of RNA polymerase (Goff *et al.*, 1984; Gottesman *et al.*, 1993). HslU and HslV were originally identified as heat shock proteins (*hsl* stands for heat shock locus), and their genes form an operon at 89 min on the chromosome (Chuang and Blattner, 1993). FtsH, whose gene is located at 69 min, is also an heat shock protein; it is transcribed together with the upstream gene, *ftsJ*, of unknown function (Herman *et al.*, 1995).

Second, ATP-dependent proteases form large oligomers of barrel-like shapes (Lupas *et al.*, 1997) which are similar to the 20S core subunit of the proteasomes, the major catalysts of ATP-dependent proteolysis in eukaryotic cells. These structures are also similar to those of chaperonins (GroEL, Braig *et al.*, 1994; thermosomes, Nitsch *et al.*, 1997; TriC/CCT, Waldmann *et al.*, 1995). Electron microscopic observations of ClpAP and HslUV revealed that their structural organizations are proteasome-like. In all of these cases, the proteolytic subunits assemble into double rings which are further flanked with rings composed of the cognate ATPase subunits (Kessel *et al.*, 1995; Rohrwild *et al.*, 1997; Lupas *et al.*, 1997). The three-dimentional structures of

several core complexes have been determined, and the three classes of proteases indeed have similar architecture (Löwe *et al.*, 1995; Groll *et al.*, 1997; Bochtler *et al.*, 1997). A ring-shaped structure of FtsH, as revealed by electron microscopy, has been reported (Shotland *et al.*, 1997), although the exact subunit assembly of FtsH is unknown.

The third characteristic shared by the ATP-dependent proteases is that they seem to catalyze multiple endo-proteolysis in processive manners, releasing short peptides. Such products could then be degraded further by energy-independent proteases/peptidases (Maurizi, 1987; van Melderen *et al.*, 1996; Thompson *et al.*, 1994). Interestingly, the proteolytic reaction by the proteasomes and HslUV seem to occur within the interior of the ring-shaped structure, since their active sites are located in the central cavities (Löwe *et al.*, 1995, Groll *et al.*, 1997, Bochtler *et al.*, 1997).

Finally, there are an increasing number of evidence suggesting that the ATP-dependent proteases possess chaperone-like activities (see below for details). It seems that energy-dependent proteases interact dynamically with their substrate proteins.

However, it must be stated that the role of ATP in protein degradation is only poorly understood. ATP hydrolysis is not required for the peptide bond cleavage itself. ATP may instead be used in the processes of recognition, unfolding and translocation of the substrate protein for initiation and continuation of proteolytic reactions.

1. Lon

The first identified ATP-dependent protease in all organisms was the *E. coli* Lon (La) protease. Lon, consisting of 4 identical subunits of 87 kDa (Chung *et al.*, 1981), is a serine protease but has no sequence similarity with the classical serine proteases (Amerik *et al.*, 1991). Lon is a major protease that degrades abnormal proteins. In *lon* mutants, proteins containing abnormal amino acids, as well as prematurely

terminated polypeptides are stabilized (Maurizi *et al.*, 1985). It also has a role in degradation of some unstable regulatory proteins, including SulA (cell division inhibitor; Mizusawa and Gottesman, 1983), RcsA (the transcriptional activator of capsular polysaccharide genes; Torres-Cabassa and Gottesman, 1987), CcdA (the antidote protein of the killing system carried by the F plasmid; van Melderen *et al.*, 1994), and λ N protein (anti-termination factor; Gottesman *et al.*, 1981). Lon homologs has been found from bacteria to human; in eukaryotes, they are located in the mitochondrial matrix space (Wang *et al.*, 1993; Suzuki *et al.*, 1994).

2. ClpAP

ClpAP (Ti) consists of two subunits, ClpA regulatory subunit of 83 kDa with ATPase activity and ClpP proteolytic subunit of 23 kDa. ClpA is a member of the Clp family proteins present in both prokaryotic and eukaryotic organisms. They include ClpA, ClpB, ClpC, ClpX, and ClpY. The ClpA/B/C subfamily proteins contain two highly conserved domains with an ATP-binding sequence. The ClpX/Y subfamily proteins have a single ClpA/B/C-type ATP binding-site. ClpP homologs forms a unique family of serine proteases (Maurizi *et al.*, 1990). ClpAP was reported to act in the "N-end rule" pathway, degrading proteins with some destabilizing N-terminal residues (Tobias *et al.*, 1991). ClpAP also degrades MazE protein, an antidote protein in a post-segregational killing system (Aizenman *et al.*, 1996). Although the ClpP subunit alone can degrade short fluorogenic peptides (Woo *et al.*, 1989), but it cannot degrade large proteins, such as casein, without ClpA and ATP hydrolysis (Hwang *et al.*, 1988). ATP-binding to ClpA promotes its self-association and the formation of the ClpA-ClpP complex (Maurizi, 1991).

3. ClpXP

ClpX (46 kDa) and ClpP associate with each other to form the ClpXP ATP-dependent protease (Gottesman *et al.*, 1993; Wojtkowiak *et al.*, 1993). The ClpXP degrades the

replication initiator O protein of phage λ (Gottesman *et al.*, 1993; Wojtkowiak *et al.*, 1993), the Phd protein of the P1 plasmid (Lehnherr and Yarmolinsky, 1995), mutant Mu phage repressor (Laachouch *et al.*, 1996), and σ^S, the stationary-phase σ factor of *E. coli* (Schweder *et al.*, 1996).

4. HslUV

HslV (ClpQ) is a 19 kDa protein having sequence homology with β-type subunits of the 20S proteasomes, and HslU (ClpY) is a 50 kDa ATPase subunit; they together function as an ATP-dependent protease. HslU belongs to the ClpX/Y family having about 50 % sequence identity with ClpX. Like the archaebacterial proteasome, the N-terminal Thr residue of HslV is the protease active site (Missiakas *et al.*, 1996; Bochtler *et al.*, 1997). The inhibitor specificity as well as the sequence and structural similarity between archaebacterial proteasome and HslV suggests that HslV can be regarded as a bacterial counterpart of the proteasome (Rohrwild *et al.*, 1996; Bochtler *et al.*, 1997). HslV has a weak peptidase activity, and HslU stimulates peptide hydrolysis by HslV in the presence of ATP (Yoo *et al.*, 1996). HslUV has been shown to be involved in degradation of some misfolded proteins *in vivo* (Missiakas *et al.*, 1996).

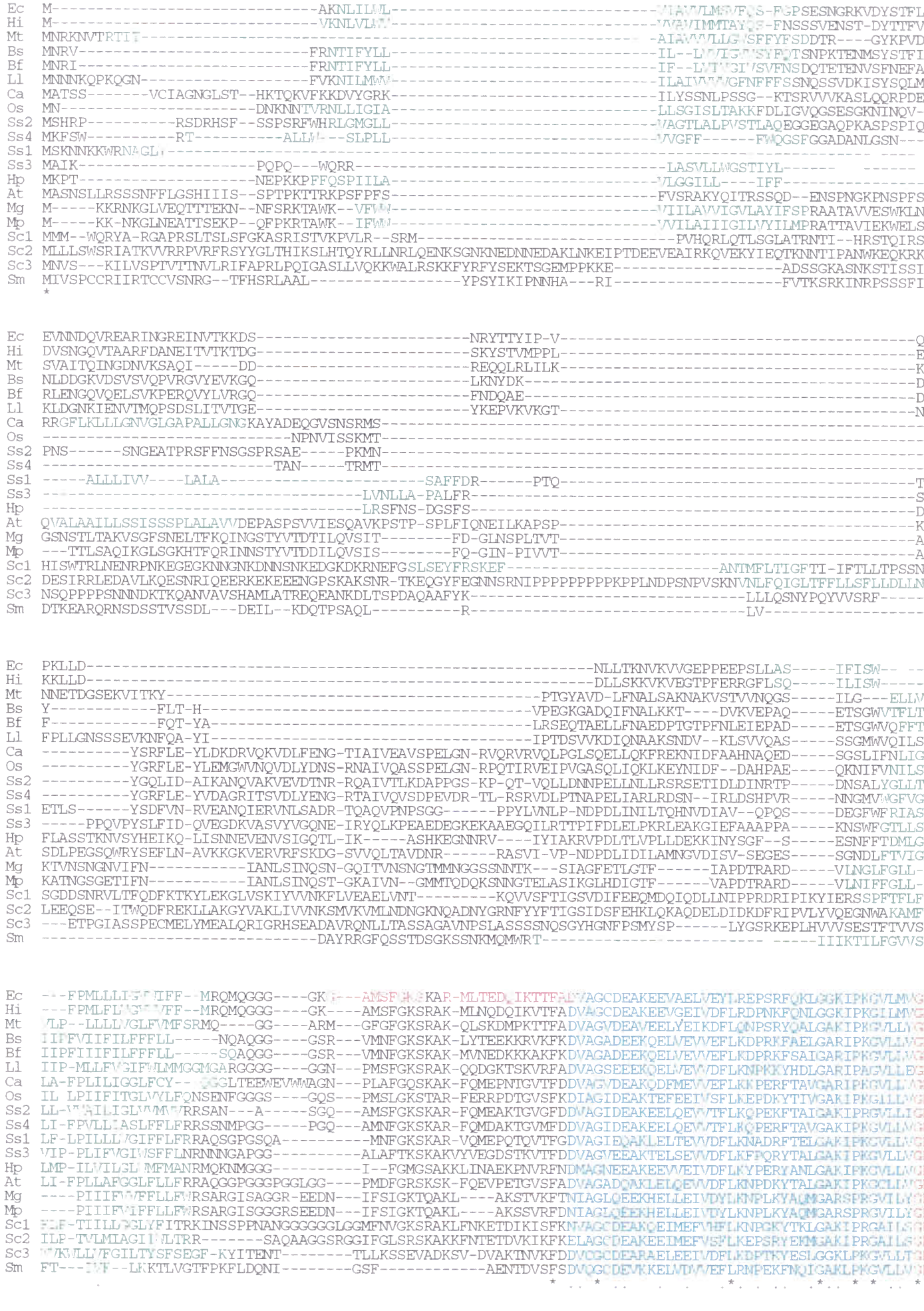
III. The FtsH family proteins

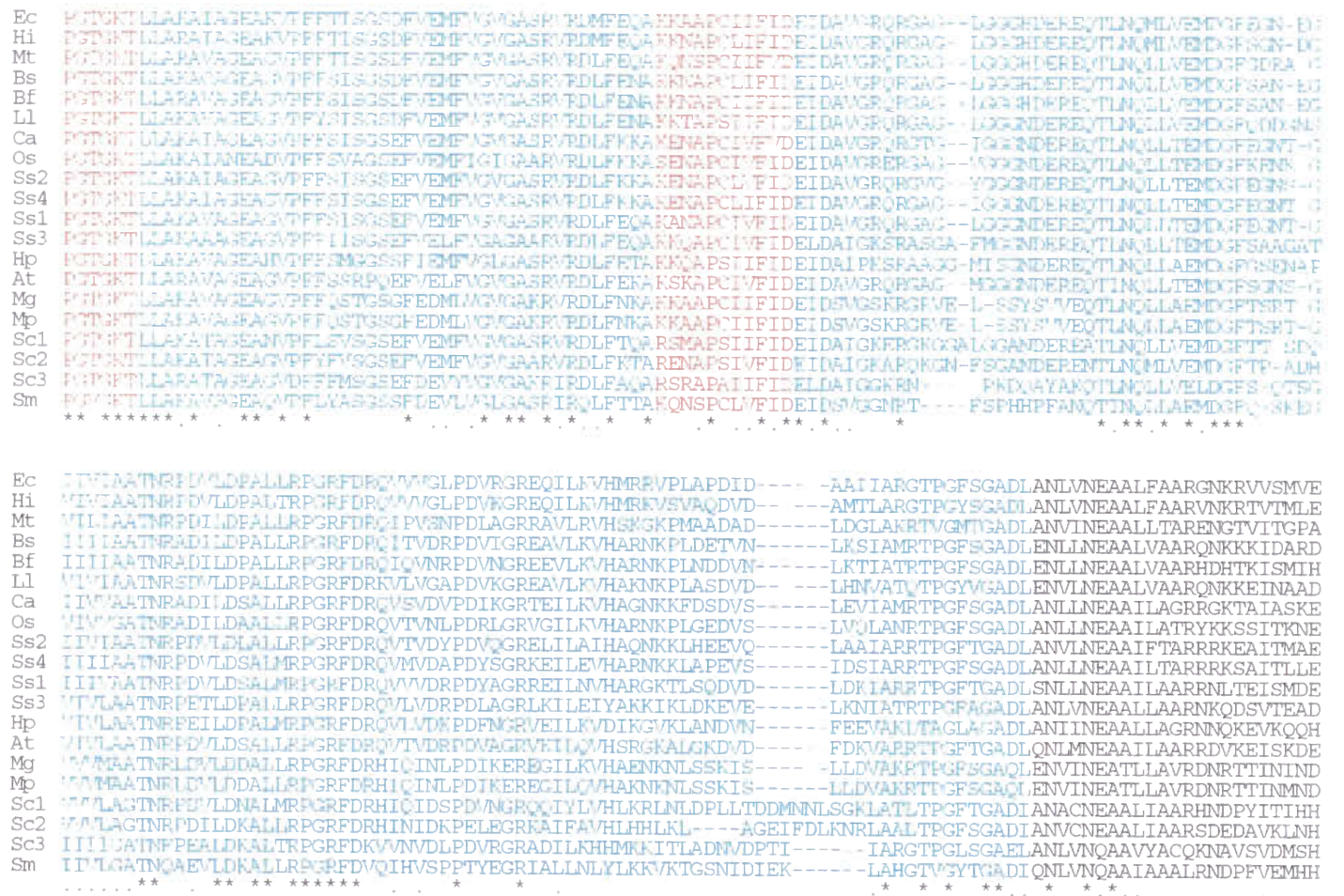
The *E. coli* FtsH protein has two transmembrane segments in the N-terminal region, whereas its cytosolic domain belongs to the AAA (ATPase associated with diverse cellular activities) family of ATPases, whose members are widely found among eukaryotes and prokaryotes (Tomoyasu *et al.*, 1993a). Although the AAA family proteins share conserved AAA domains of about 200 amino acid residues (Fig. 1; light blue), their physiological functions appear quite diverse; they include regulation of cell cycle, vesicular fusion, biogenesis of organelles, regulation of transcription, and protein degradation (Confalonieri and Duguet, 1995). FtsH and its homologs also contain a sequence motif of the zinc-metalloproteinase active site.

FtsH homologs exist widely among prokaryotes as well as organelles of the supposed prokaryotic origin in eukaryotes. Interestingly, the results of genome sequencing projects revealed that some organisms have multiple FtsH homologs. For example, three FtsH homologs exist within the mitochondria of *Saccharomyces cerevisiae* (Schnall *et al.*, 1994), and *Synechocystis sp.*, a cyanobacterium, has four FtsH homologs (Kaneko *et al.*, 1996). Red pepper fruits (*Capsicum annuum*) FtsH homolog (Pftf) has been shown to be localized in the plastid (Hugueney, 1995). The AAA domains (Fig. 1; light blue) are highly conserved among the FtsH homologs, and the conservation extends into regions of about 200 amino acids following the AAA domains. They all have conserved zinc-binding motif (HEXXH) (blue), ATP binding motif A (G(A)XXXXG(A)K(R, H)X_{0-1}T(S, R, K, H); Chin *et al.*, 1988) and motif B (R(K, H)X5-8ΦXΦΦD(E); where Φ represents any hydrophobic amino acid residues (I, V, L, M, W, or F) (red). Akiyama *et al.* (1994b) pointed out the exsitence of an additional set of ATP binding motifs (ATP-binding sequence 2) (Fig. 1, magenta) in amino acid sequence of *E. coli* FtsH, although it is not conserved in other FtsH homologs. Most of the FtsH homologs have two putative transmembrane segments (green) at their N-termini with exceptions of Yta11p (Yme1p) and *Scistosoma mansoni* FtsH having only one transmembrane region.

1. *Escherichia coli* FtsH

E. coli FtsH (also called HflB or TolZ) has N-terminally located two transmembrane segments and a large cytoplasmic domain which contains the AAA domain and a zinc metalloproteinase signature sequence (HEXXH) (Tomoyasu *et al.*, 1993a, b; Tomoyasu *et al.*, 1995). The N-terminal transmembrane region of FtsH mediates a homo-oligomeric interaction of this protein (Akiyama *et al.*, 1995). FtsH is the only protease that is essential for growth of *E. coli* (Akiyama *et al.*, 1994a). The *ftsH* gene had originally been defined by a temperature-sensitive cell division mutation (filamentous temperature-sensitive) (Santos and Almeida, 1975). However, it was





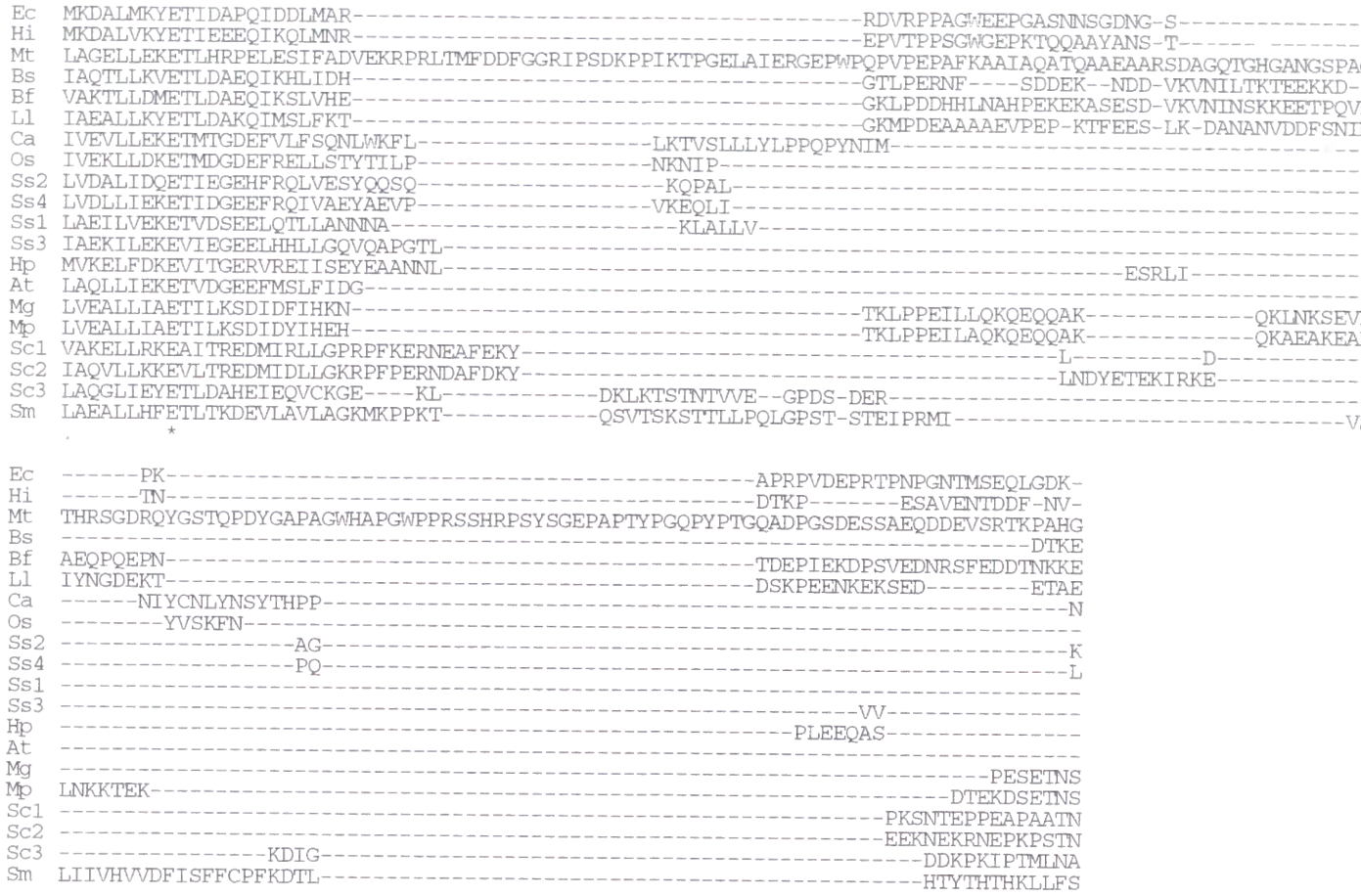


Fig. 1 Amino acid sequence comparison of FtsH homologs. The alignment includes FtsH from *Escherichia coli* (Ec; accession number M83138), *Haemophilus influenzae* (Hi; U32824), *Mycobacterium tuberculosis* (Mt; Z95557), *Bacillus subtilis* (Bs; D26185), *Bacillus firmus* (Bf; U61844), *Lactococcus lactis* (Tma) (Ll; X69123), *Capsicum annuum* (Pftf) (Ca; X80755), *Odontella sinensis* (Os; Z67753), *Synechocystis sp.* (Ss1~Ss4; D90902, D90904, D90906, and D64000), *Helicobacter pylori* (Hp; U59452), *Arabidopsis thaliana* (At; X99808), *Mycoplasma genitalium* (Mg; U39732), *Mycoplasma pneumoniae* (Mp; U00089), *Saccharomyces cerevisiae* (Sc1~Sc3; X76643 (*YTA10*), L14616 (*YTA11*), X81068 (*YTA12*)), and *Scistosoma mansoni* (Sm; Z29947). Asterisks show identical residues, and dots show similar residues. Putative transmembrane regions are shown in green. The AAA domains, ATP-binding sequences, and zinc-binding sites are indicated by light blue, red, and blue, respectively. The ATP-binding sequence 2 (Akiyama *et al.*, 1994b) found in *E. coli* FtsH is shown in magenta.

later shown that the original "*ftsH*" mutant contained two mutations, one in *ftsH* and the other in *ftsI*, the latter mutation affecting penicillin-binding protein 3 being primarily responsible for the cell division defect. The *ftsH* mutation alone caused only slight cell elongation (Begg *et al.*, 1992). In 1993, Herman *et al.* reported that *hflB* mutation is an allele of the *ftsH* gene. The *hflB* mutations had been isolated as mutations causing high frequency lysogenization of λ phage (*hfl* stands for high frequency of lysogeny) (Banuett *et al.*, 1986). In *hflB* mutants, unstable λ CII protein, a transcription factor necessary for lysogeny establishment (see below), is stabilized (Banuett *et al.*, 1986). Thus, connection between FtsH and proteolysis stemmed from the Hfl studies. Later, our group (in this study; Kihara *et al.*, 1997) and Shotland *et al.* (1997) independently showed that purified FtsH degrades CII in an ATP-dependent manner *in vitro*. In addition, proteolytic roles of FtsH have been established for SecY (in this study; Kihara *et al.*, 1995; Akiyama *et al.*, 1996a), subunit *a* of the F_0 part of H^+-ATPase (Akiyama *et al.*, 1996b) and the σ^{32} RNA polymerase subunit (Tomoyasu *et al.*, 1995; Herman *et al.*, 1995).

Two other genetic screenings resulted in the isolation of *ftsH* mutants. In one approach, Akiyama *et al.* (1994a, b) obatained *std* mutants. Integral membrane proteins have hydrophobic stretches (transmembrane segments) embedded directly into the lipid phase of the membrane. A stop transfer sequence is thought to act to stop the movement of a polypeptide segment during translocation of the polypeptide chain across the membrane, generating a membrane anchored region of the protein. A transmembrane segment thus generated has its carboxy terminal side remaining in the cytoplasm. Akiyama *et al.* (1994a, b) used a SecY-PhoA fusion protein, in which PhoA was attached to the sixth cytoplasmic region of SecY. The localization-dependent folding property of PhoA was used to monitor its disposition in the cell. It was found that a greater proportion of the PhoA domain was exported in the *ftsH* mutants. This phenotype was called Std, stop transfer defective. Evidence suggested that the Std phenotype of the *ftsH* mutation is not solely ascribable to the proteolytic function

of FtsH. Thus, it was proposed that FtsH somehow mediates the stop transfer event that should normally occur for the hydrophobic stretch preceding the PhoA region. Alternatively, FtsH might interact with the PhoA moiety to retain it in the cytoplasm. These studies provided the foundation for the proposal that FtsH has a chaperone-like function (see below). Some export defects of Bla and OmpA were also noted for the *ftsH* mutants (Akiyama *et al.*, 1994a, b). However, the exact role played by FtsH in the protein translocation/integration processes remains unknown.

Qu *et al.* (1996) isolated a mutation called *tolZ21*, which turned out to be an allele of *ftsH*, as causing tolerance to colicins E2, E3, D, Ia, and Ib (Tol^-). The *tolZ* mutant can grow on glucose as a carbon source but not on succinate or other nonfermentable carbon sources (Nfc^-). It is not known how the *tolZ21* alteration in FtsH (His^{418} to Tyr substitution within the zinc-binding motif, $H_{415}EXXH$) causes the Tol^- and Nef^- phenotypes. The *tolZ21* mutation seems to inactivate FtsH, and a suppressor mutation (*sfhC*) proved to be required for the cell survival (Qu *et al.*, 1996); *sfhC* is in the *fabZ* gene involved in fatty acid biosynthesis (Ogura, personal communication).

2. *Bacillus subtilis* FtsH

A *ftsH* mutant of *B. subtilis*, a gram-positive bacterium, was first isolated as a salt-sensitive mutant (Gleisler and Schumann, 1993). The *B. subtilis ftsH* gene has been sequenced through the genome sequencing project (Ogasawara *et al.*, 1994). Although *E. coli ftsH* is essential for growth, *ftsH* disruption mutants of *B. subtilis* are viable, exhibiting the filamentous morphology with increased sensitivity to heat and salt stresses (Deuerling *et al.*, 1997). The *ftsH* gene is transiently induced after osmotic and temperature upshift (Deuerling *et al.*, 1995). After entry into a stational phase, *ftsH* null-mutant cannot sporulate, and secretion of many exoproteins is impaired (Deuerling *et al.*, 1997). Many of these phenotypes may be explained in terms of the reduced amounts of SpoOA protein, which is a transcription activator (Deuerling *et al.*, 1997). SpoVM, a sporulation-related small protein, is a substrate of FtsH (Cutting

et al., 1997).

3. *Lactococcus lactis* FtsH (Tma)

L. lactis is a gram-positive bacterium which is used widely for industrial homo-lactic fermentation. *L. lactis ftsH* is discovered as a gene adjacent to *hpt*, which encodes a guanine phosphoribosyltransferase. *L. lactis* FtsH is 47% idential to *E. coli* FtsH and can substitute for *E. coli* FtsH in *E. coli* cells (Nilsson *et al.*, 1994). *ftsH* may not be essential in *L. lactis*, since an insertion mutant within the *ftsH* gene renders cells cold- and temperature-sensitive, while capable of growing at 30°C (Nilsson *et al.*, 1994). This mutant shows salt sensitivity, like the *B. subtilis ftsH* mutant.

4. *Saccharomyces cerevisiae* FtsH homologs

In *S. cerevisiae*, YTA proteins (Yeast Tat binding Analogs) have been identified as proteins having the conserved ATP-binding sequences (Schnall *et al.*, 1994). Among them, three mitochondrial proteins, Yta10p (Afg3p), Yta11p (Yme1p), and Yta12p (Rca1p), exhibit high sequence homology to the *E. coli* FtsH protein. Yta10p and Yta12p form a complex in the mitochondrial inner membrane with their large domains exposed to the matrix space (Arlt *et al.*, 1996). Yta11p has its main domain exposed to the intermembrane space (Leonhard *et al.*, 1996). These proteins have roles in proteolysis in mitochondria. The mitochondrial genome of *S. cerevisiae* encodes eight proteins; ribosomal Val1 protein, subunits I, II, and III of the cytochrome c oxidase (Cox1, Cox2, and Cox3), apocytochrome b (Cob), and subunit 6, 8, and 9 of the ATP synthase (Su6, Su8, and Su9). These proteins are unstable in the absence of a supply of their nuclear-encoded partners. Yta10p (Afg3p) is involved in the degradation of Cox1, Cox3, Cob, Su6, Su8, and Su9 (Guélin *et al.*, 1996). On the other hand, non-assembled Cox2 seems to be a substrate of Yme1p (Yta11p) because its degradation is partially suppressed by disruption of the *YME1* gene (Nakai *et al.*, 1995; Pearce and Sherman, 1995). Leonhard *et al.* (1996) showed that the Yta10p/Yta12p complex

degrades a model protein (Su9(1-66)-pCOXII(1-74)-DHFR) spanning the inner membrane of mitochondria from the matrix side, while Yme1p (Yta11p) degrades it from the intermembrane side. As described in the next section, it was proposed that the Yta10p/Yta12p complex possesses a chaperone-like activity.

The *YME1* gene (yeast mitochondrial escape) has also been identified by a screen for mutants in which escape of mitochondrial DNA to the nucleus is evident (Thorsness and Fox, 1993; Thorsness *et al.*, 1993). In addition to an increased rate of DNA escape from mitochondria, *yme1* mutants shows several other phenotypes including temperature-sensitive respiration, cold-sensitive growth on rich glucose medium, and synthetic lethality with a rho$^-$ (cytoplasmic petite) mutaion. It is not known why these diverse phenotypes are caused in the absence of the Yme1p function.

IV. Chaperone activities of ATP-dependent proteases

ATP-dependent proteases or their ATPase subunits share some properties with molecular chaperones (for a review, see Suzuki *et al.*, 1997). ClpA can substitute for the DnaK/DnaJ/GrpE chaperones, in the *in vitro* activation of RepA replication initiator protein of the plasmid P1, by dissociating its dimer into active monomer (Wickner *et al.*, 1994). ClpA can also target RepA for degradation by ClpP. In addition, ClpA protects luciferase from irreversible heat inactivation (Wickner *et al.*, 1994). ClpX has also been shown to possess chaperone activities. ClpX mediates the disassembly of the MuA transposase from the MuA-Mu DNA complex *in vitro*, an initial process of Mu DNA replication (Levchenko *et al.*, 1995). Bacteriophage Mu cannot replicate in the *clpX* mutant but can do so in the *clpP* mutant, indicating that ClpX acts independently from ClpP in supporting Mu replication (Mhammedi-Alaoui *et al.*, 1994). ClpX can also protect the λO protein from heat-induced aggregation and can disaggregate the preformed aggregates (Wawrzynow *et al.*, 1995).

A mutation either in *RCA1* (*Yta12*) or in *AFG3* (*Yta10*) causes a pleiotropic deficiency in mitchondrial respiration functions (Tzagoloff *et al.*, 1994; Guélin *et al.*, 1994; Paul

and Tzagoloff, 1995). Paul and Tzagoloff (1995) and Rep *et al.* (1996) showed that assembly of the F_1 ATPase was impaired in these mutants. Subunit 9 of the F_0 moiety of ATPase cannot oligomerize in the absence of Yta10p or Yta12p (Arlt *et al.*, 1996). These results indicated that the Yta10-12 complex is somehow involved in the assembly of these subunits into the functional enzymes. The respiratory deficiency and the impaired oligomerization of subunit 9 in Δ*yta10* and of Δ*yta12* cells can be restored by expression of the protease active-site mutant of Yta10p and Yta12p, respectively (Arlt *et al.*, 1996). In addition, overproduction of yeast mitochondrial Lon (PIM1p) as well as its protease active-site mutant, Lon S104A, can also restore the respiratory defect of the *yta10 yta12* double mutant (Rep *et al.*, 1996). The assembly defect of cytochrome c oxidase and F_1-F_0 ATPase can also be suppressed by overproduction of Lon (Rep *et al.*, 1996). It was thus suggested that the Yta proteins have a chaperone-like role in the assembly of mitochondrial protein complexes, and the mitochondrial Lon protein can substitute for them to certain extents.

As already discussed, the Std phenotype of the *ftsH* mutants suggested that *E. coli* FtsH has a chaperone-like activity. The growth retardation of the *ftsH* mutants can be partially suppressed by overproduction of GroEL/GroES or HtpG (Shirai *et al.*, 1996). The Std phenotype of the *ftsH* mutant is alleviated by overproduction of HtpG (Hsp90) (Shirai *et al.*, 1996). The defects in export of Bla and OmpA (Akiyama *et al.*, 1994a) are also alleviated by overproduction of GroEL/GroES (Shirai *et al.*, 1996). These results suggest functional overlaps between FtsH and some chaperone proteins (GorEL/GroES and HtpG). FtsH can bind to a denatured form of PhoA but not to the native enzyme (Akiyama *et al.*, submitted). Interesingly, this binding is not accompanied by degradation of the bound PhoA protein. Thus, like other molecular chaperones, FtsH seems to have an ability to bind to a denatured protein, and such a binding activity can be independent of the proteolytic function of this protein.

V. The history of the λ CII protein and the host Hfl functions

After infection of bacteriophage λ to the host *E. coli* cells, the λ genome undergoes either integration as a prophage into the host chromosome (lysogenization) or replication to form progeny phages (lytic growth). The λ CII protein is a key determinant in the lysis-lysogenization decision. It is a transcription regulator that activates three promoters for the λ genes essential for lysogeny establishment: P_{RE}, P_I, and P_{AQ}. The P_{RE} promoter controls the "establishment" mode of transcription of the *cI* gene for the major repressor of λ. The transcription initiated at P_I leads to the production of the *int* gene product, the integrase for prophage insertion (Shimatake and Rosenberg, 1981). The antisense mRNA initiated at P_{AQ} quenches translation of the Q protein (Ho and Rosenberg 1985; Hoopes and McClure, 1985), an anti-terminator protein that is necessary for the expression of all the late genes for lytic growth. Therefore, if CII is abundant, the lysogenic pathway is chosen; whereas the lytic pathway is preferred under the low CII concentration. Intracellular concentration of CII is controlled not only at the level of transcription, but at the level of protein stability. The CII protein is very unstable and degraded with a half-life of about 2 min in the wild-type cells (Gottesman *et al.*, 1981; Hoyt *et al.*, 1982). Thus, the stability control of CII is critical for the decision for lysogenization vs lysis. Belfort and Wulff (1971) first isolated an *E. coli* mutant (*hfl-1* or *hflA1*; high frequency lysogenization) which allowed high frequency lysogenization of λ. Gautsch and Wulff (1974) obtained additional 6 *hfl* mutants. Five of them as well as the *hfl-1* mutaion fell into a single complementation group designated *hflA* (Gautsch and Wulff, 1974). The remaining mutation (*hflB29*) was mapped in a different locus, *hflB*, now known as an allele of *ftsH* (Herman *et al.*, 1993). Sequencing analysis revealed subsequently that the *hflA* locus consists of three genes, designated *hflX*, *hflK*, and *hflC* (Noble *et al.*, 1993). Among them, mutations in the *hflK* or *hflC* caused the Hfl phenotype (Banuett and Herskowitz, 1987). HflX has a sequence motif typical of GTP-binding proteins (Noble *et al.*, 1993), but its funcition is not known. HflK and HflC are membrane proteins and form a complex (HflKC) (Zorick and Echols, 1991;

Cheng *et al.*, 1988). The unstable λ CII protein is stabilized in *hflA* mutants (Hoyt *et al.*, 1982; Banuett *et al.*, 1986), resulting in the Hfl phenotype. Cheng *et al.* (1988) reported that a purified HflKC preparation possessed a CII-degrading activity which was inhibited by PMSF, an inhibitor of serine proteases. In accordance with this observation, it was claimed that HflC contains a sequence motif similar to that found in the active site region of ClpP (Noble *et al.*, 1993). It has been believed that FtsH and HflKC participate in independent pathways of degradation of the CII protein for the following reasons (Banuett *et al.*, 1986; Herman *et al.*, 1993). First, *ftsH* mutations (*ftsH1* or *hflB29*) increase the frequency of lysogenization even in an *hflA* null background (Banuett *et al.*, 1986; Herman *et al.*, 1993). Second, overproduction of FtsH in the *hflA*::Tn*5* cells compensates for the Hfl phenotype (Herman *et al.*, 1993).

In this study, we showed that HflKC forms a complex with FtsH. Contrary to the previous notion, we showed that HflKC, which was found to be periplasmically exposed, is not a CII-degrading protease but is rather a modulator of the FtsH protease. This regulation seems to differentiate the FtsH actions against different substrate proteins such as the membrane-bound SecY protein and cytosolic CII protein.

§ 2 Results

I. FtsH is the protease responsible for the degradation of SecY

1. Overproduced SecY is stabilized in the *ftsH* mutants

In order to identify the proteolytic system responsible for the rapid degradation of oversynthesized SecY, we introduced pKY248 (Taura *et al* , 1993), a pACYC184-derived *secY* plasmid, into several mutants affected in proteolysis, and examined stability of SecY by pulse-chase-immunoprecipitation experiments. The mutants examined were *lon*, *clpA*, *clpP*, *hflA*, *hflB*, *spp*, *ptr* and *degP*, but none of them exhibited significant stabilization of SecY (data not shown). The above tests included the *hflB29* mutant known to be defective in the degradation of the λ CII protein (Banuett

et al., 1986), and this mutation proved to be an allele of *ftsH* (Herman *et al.*, 1993), a gene encoding a membrane protein whose cytoplasmic domain belongs to an ATPase family (Tomoyasu *et al.*, 1993a).

We previously isolated another allele, *ftsH101*, of this gene through a search for "std" (stop transfer defective) mutants, in which normally cytoplasmic PhoA domain of a SecY-PhoA fusion protein was exported at an appreciable efficiency (Akiyama *et al.*, 1994a). We found that overproduced SecY was partially stabilized in this mutant cells. Whereas excess SecY molecules, pulse-labeled in the wild-type cells (Fig. 2A, lanes 1-4), were degraded down to the basal level within 5 min (lane 3), a significantly higher proportion of pulse-labeled SecY remained after chases in the *ftsH101* mutant cells after chases (lanes 7 and 8). Stability of SecY in the *ftsH1* (Ts) mutant was then studied. Pulse-chase examinations were carried out after shift to 42°C, the non-permissive temperature for this mutant. As shown in Fig. 2B (lanes 5-8), no appreciable degradation of SecY was seen up to the 20 min chase point examined. We noticed an apparent increase in initial labeling of SecY in the *ftsH* cells as compared with the *ftsH*$^+$ cells, but these differences were probably due to the rapid degradation of newly synthesized SecY under the *ftsH*$^+$ conditions (Taura *et al.*, 1993) rather than to increased synthesis rates in the mutant cells.

2. Normal FtsH function is required for degradation of excess SecY

A plasmid carrying *ftsH*$^+$ (pSTD401, with the replication system of pSC101) or its vector counterpart (pHSG575) was introduced into the *ftsH101* mutant or the *ftsH*$^+$ cells, both carrying pKY318 (a pBR322-derived *secY* plasmid). Fig. 3 depicts the pulse-chase profiles of SecY that was immunoprecipitated using a fixed total radioactivity for each sample. The initial incorporation (without chase) should reflect the balance among three factors, the rate of translation initiation, degree of chain completion and degree of degradation. As already noted, the rapid proteolysis of SecY results in reduced net incorporation of radioactivity into SecY, even if a pulse

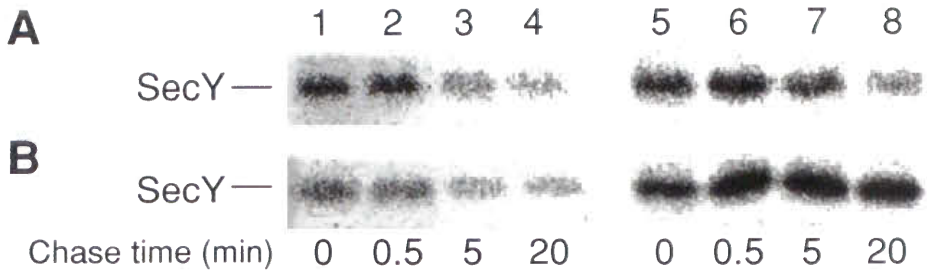


Fig. 2 Degradation of excess SecY is defective in *ftsH* mutants.
A. Cells of AK318 (wild type; lanes 1-4) and AK315 (*ftsH101*; lanes 5-8), each bearing pKY248 (p*lac-secY*) were grown at 37℃. **B**. Cells of AR796 (*ftsH*$^+$; lanes 1-4) and AR797 (*ftsH1*; lanes 5-8), each bearing pKY248 were grown at 30℃ and shifted to 42℃ 1 hr before pulse-labeling. In both **A** and **B**, plasmid-encoded *secY* was induced for 10 min, pulse-labeled with [^{35}S]methionine for 30 sec, and chased with unlabeled L-methionine for 0 (lanes 1 and 5), 0.5 (lanes 2 and 6), 5 (lanes 3 and 6), and 20 (lanes 4 and 8) min. Radioactive SecY was immunoprecipitated, subjected to SDS-PAGE, and visualized by autoradiography.

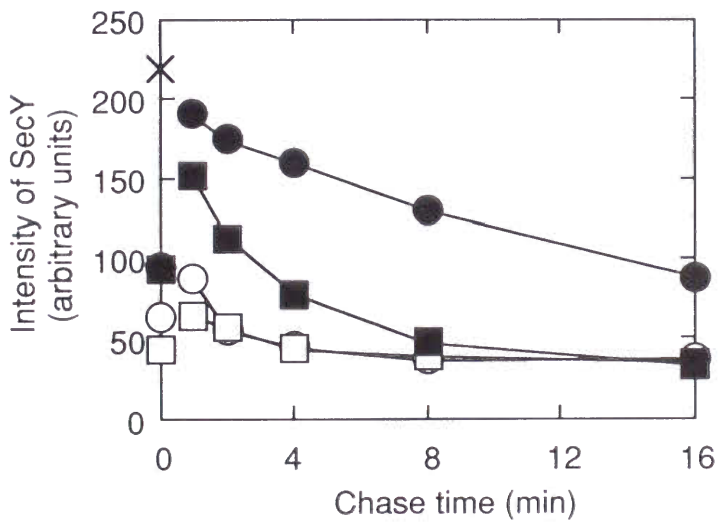


Fig. 3 Overproduction of FtsH accelerates degradation of SecY.
Cells of AK315 (*ftsH101*)/pKY318 (p*lac-secY*)/pSTD401 (p*lac-ftsH*) (open circles), AK315/pKY318/pHSG575 (vector) (closed circles), AK318 (*ftsH*$^+$)/pKY318/pSTD401 (open squares), and AK318/pKY318/pHSG575 (closed squares) were grown at 30°C and then induced with 1 mM IPTG. Cells were pulse-labeled with [^{35}S]methionine for 30 sec, and chased with unlabeled methionine for the indicated periods, followed by immunoprecipitation of SecY and SDS-PAGE. Radioactivities associated with SecY were quantitated. About 6.5 x 10^5 cpm of total cell proteins were used in each sample. The symbol (X) on the ordinate indicates a hypothetical initial labeling assuming that all the labeled chains had been completed and no degradation had taken place during translation, and obtained by extrapolating the logarithmically plotted versions of curves (closed circles) and (closed squares).

length as short as 30 sec is used (Taura *et al.*, 1993). The half-life of excess SecY was about 12 min in the *ftsH101* mutant carrying the vector plasmid (closed circles). In the presence of pSTD401, a fraction of SecY was strikingly destabilized, whereas the other fraction was not appreciably degraded (open circles). The former and the latter fractions should have represented, respectively, SecY in excess over SecE and SecY that formed a stable complex with SecE (Taura *et al.*, 1993). From the degradation phase, we estimated that the half-life of excess SecY is about 1 min by assuming that the synthesis rate itself equals to that obtained by extrapolating the degradation phases of the upper two curves of Fig. 3. Moreover, overproduction of $FtsH^+$ in the pKY318-bearing *ftsH*$^+$ cells accelerated the degradation of excess SecY, shortening its estimated initial half-life from about 2.5 min (closed squares) with the vector plasmid to immeasurable (open squares).

These results show that the *ftsH101* mutation is recessive with respect to the stabilization of excess SecY; the stabilization is due to a loss of FtsH function rather than to active interference of the mutant form of FtsH with the degradation. The enhanced SecY degradation in the presence of excess FtsH suggest that FtsH is rate-limiting in degradation of excess SecY. This is consistent with the results obtained with the insertion mutaions reducing the *ftsH* expression and stabilizing SecY (see below).

3. Mutations in *ftsH* suppress the temperature-sensitive export defect of the *secY24* mutant by stabilizing the mutant protein

The *secY24* mutation, a Gly^{240} to Asp alteration in cytoplasmic domain 4 of SecY (Shiba *et al.*, 1984), weakens SecY-SecE interaction and causes a temperature-sensitive export defect (Baba *et al.*, 1994). Pulse-chase experiments showed that the chromosomally encoded $SecY^+$ (without overproduction) was stable both at 30 and 42°C (Baba *et al.*, 1994; see also Fig. 7, open circles). The *secY24* mutant protein was stable at 30°C but degraded with a half-life of about 20 min upon exposure to 42°C

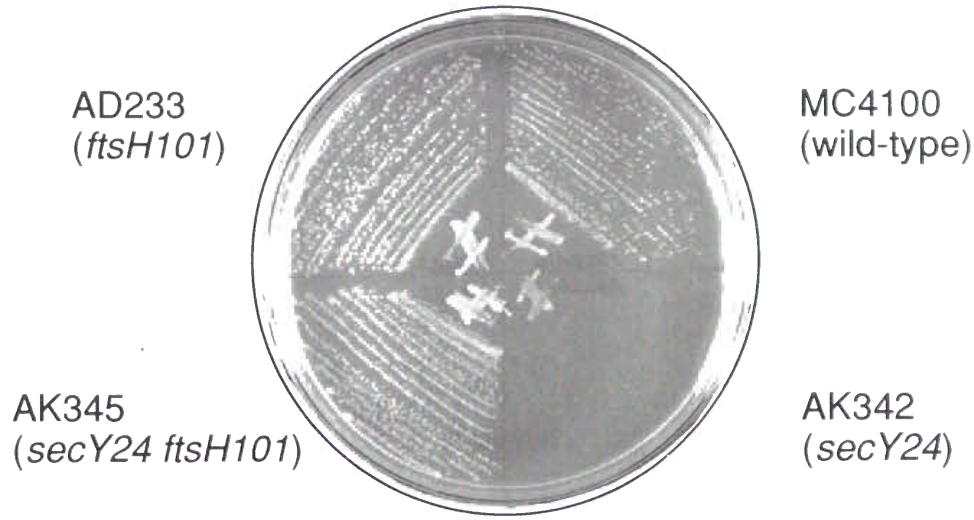


Fig. 4 Suppression of the temperature-sensitive growth of the *secY24* mutant by the *ftsH101* mutation.
Strains MC4100 (*secY*$^+$ *ftsH*$^+$), AD233 (*ftsH101*), AK342 (*secY24 ftsH*$^+$) and AK345 (*secY24 ftsH101*) were streaked on minimalE-glucose agar plate and incubated at 42°C for 18 hr.

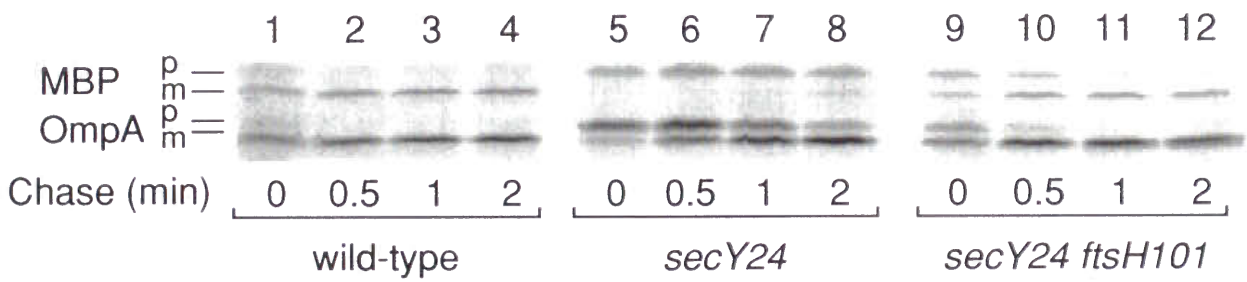


Fig. 5 The *ftsH101* mutation suppresses the temperature-sensitive export defect of the *secY24* mutant.
Cells of MC4100 (*secY*$^+$*ftsH*$^+$; lanes 1-4), AK342 (*secY24 ftsH*$^+$; lanes 5-8), and AK345 (*secY24 ftsH101*; lanes 9-12) were grown first at 30°C and then shifted to 42°C for 1 hr. Cells were pulse-labeled with [^{35}S]methionine for 0.5 min and chased for the indicated periods. MBP and OmpA were immunoprecipitated. p and m indicate precursor and mature forms, respectively.

(Baba *et al.*, 1994; see also Fig. 7, closed circles).

When the *ftsH101* mutation was combined with the *secY24* mutation, the resulting strain (AK345) was found to be able to grow at 42°C on minimal E agar, on which the *secY24* single mutant (AK342) could not grow at this temperature (Fig. 4). In other words, *ftsH101* suppressed *secY24* with respect to the temperature sensitivity. To examine whether the protein export defect was suppressed as well, MBP and OmpA proteins were pulse-chased and proportions of precursor species were measured (Fig. 5). The *ftsH101* mutation indeed improved the efficiencies of MBP and OmpA exports in the presence of *secY24* at 42°C (compare *ftsH101 secY24* double mutant shown in lanes 9-12 with *secY24* mutant shown in lanes 5-8), although suppression of the defect was not complete (compare lanes 9-12 with *secY*$^+$ shown in lanes 1-4).

The abundance of SecY before and after the temperature shift was examined by immunoblotting (Fig. 6). SecY abundance in the wild-type cells was not affected by the temperatures examined (open column). SecY in the *secY24* mutant cells was about 70% and 23% of the wild-type level, at 30°C and upon 1 hr exposure to 42°C (closed column), respectively. The *secY24 ftsH101* double mutant contained about 90% and 60% of the wild-type value at 30°C and at 42°C (hatched column), respectively. Thus, SecY24 is significantly stabilized in the double mutant. We also combined the *secY24* and the *ftsH1* mutations. The *ftsH1* mutation indeed stabilized the chromosomally encoded SecY24 protein at 42°C (Fig. 7). In contrast to the *secY24* single mutant in which SecY24 was degraded gradually (Fig. 7, closed circles), the *secY24 ftsH1* double mutant (open squares) contained SecY24 that was as stable as SecY$^+$ in the wild-type cells (open circles).

4. A major class of SecY-stabilizing mutations affect *ftsH*

We isolated mutants defective in degradation of SecY24 as well as of oversynthesized wild-type SecY. We first selected spontaneous revertants, capable

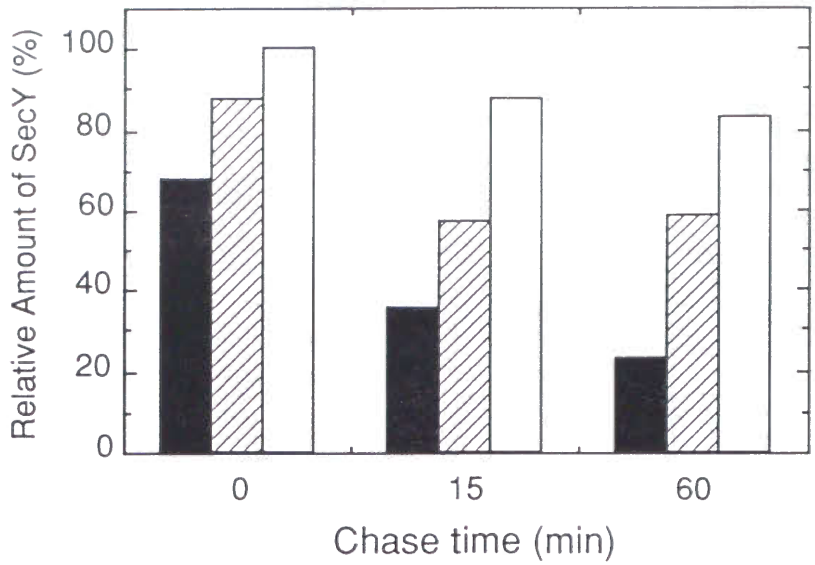


Fig. 6 The SecY24 mutant protein (chromosomal level) is stabilized in the *ftsH101* mutant. Cells of AK342 (*secY24 ftsH*$^+$; closed column), AK345 (*secY24 ftsH101*; hatched column), and MC4100 (*secY*$^+$*ftsH*$^+$; open column) were grown at 30°C, shifted to 42°C and, at the indicated time points after the shift, portions of cultures were treated with TCA. Two fixed amounts (2 0 ng and 60 ng) of SDS-solubilized proteins were separated by SDS-PAGE, western-blotted and stained with antibodies against SecY. Intensities of SecY relative to that in the wild-type cells grown at 30°C are graphically depicted.

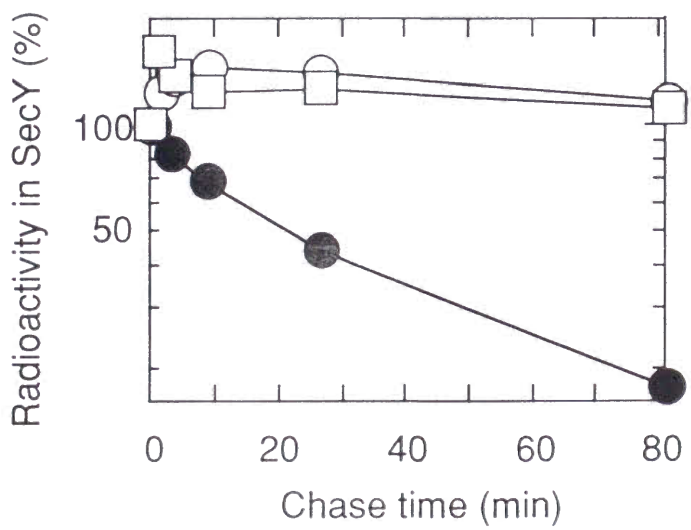


Fig. 7 The SecY24 mutant protein (chromosomal level) is stabilized by the *ftsH1* mutation. Cells of MC4100 (wild-type; open circles), AK420 (*secY24*; closed circles) and AK421 (*secY24 ftsH1*; open squares) were grown at 30°C and then shifted to 42°C. At 1 hour after temperature shift, cells were pulse-labeled with [^{35}S]methionine for 30 sec and chased with unlabeled methionine for 0, 1, 3, 9, 27 and 81 min. SecY was immunoprecipitated and separated by SDS-PAGE. Values relative to that at 0 min chase point are shown for each curve.

of growing at 42℃, from the *secY24* mutant. Those revertants in which the temperature-sensitivity was suppressed by stabilization of SecY24 were identified by examining β-galactosidase activity of the SecY-LacZα fusion protein. Model experiments showed that stability of the LacZα portion was dictated by the stability of the SecY part of the fusion protein (see also Homma *et al.*, 1995). Many of the mutants thus obtained exhibited cold-sensitive growth. All of the independently isolated cold-sensitive mutants tested were complemented by pSTD401 (*ftsH*$^+$), and mapped in the *ftsH* region of the chromosome by P1 transduction experiments. These mutations conferred cold-sensitivity when they were transduced into the *secY*$^+$ background as well. We carried out pulse-chase experiments to ensure that they indeed stabilized oversynthesized SecY (Fig. 8). In wild-type cells, the fraction of SecY molecules that were excess over SecE were degraded with a half-life of about 2 min (closed circles). However, in the mutant cells, the unstable SecY fraction was stabilized such that it remained even at the 20 min chase point (*ftsJ-101*::IS*10*L, open circles; *ftsH102*, open triangles; *zgj-525*::IS*1*A, open squares; see below for nomenclature of the mutations). Similar retardation of SecY degradation was also seen in other mutants that passed our screenings (data not shown).

We cloned the *ftsH* region from each of the mutant chromosomes and determined the nucleotide sequence. One of them, named *ftsH102*, had a single T^{1432} to G base change in *ftsH* that should result in a Leu^{189} to Trp change in the protein product (Tomoyasu *et al.*, 1993a). The rest of the mutants proved to have insertions of IS*1*A (Halling *et al.*, 1982) or IS*10* (Umeda and Ohtsubo, 1991) in the promoter-distal region of *ftsJ* or in the intergenic region between *ftsJ* and *ftsH* (Fig. 9). It was reported that *ftsJ* and *ftsH* form an operon (Herman *et al.*, 1995). We investigated the synthesis level of FtsH in these mutant cells by pulse-labeling and immunoprecipitation experiments (Fig. 10). Consistent with the report that FtsH is heat inducible (Herman *et al.*, 1995), the synthesis of FtsH was transiently increased about three fold upon temperature shift from 37°C to 42°C in wild-type cells (Fig. 10, compare hatched

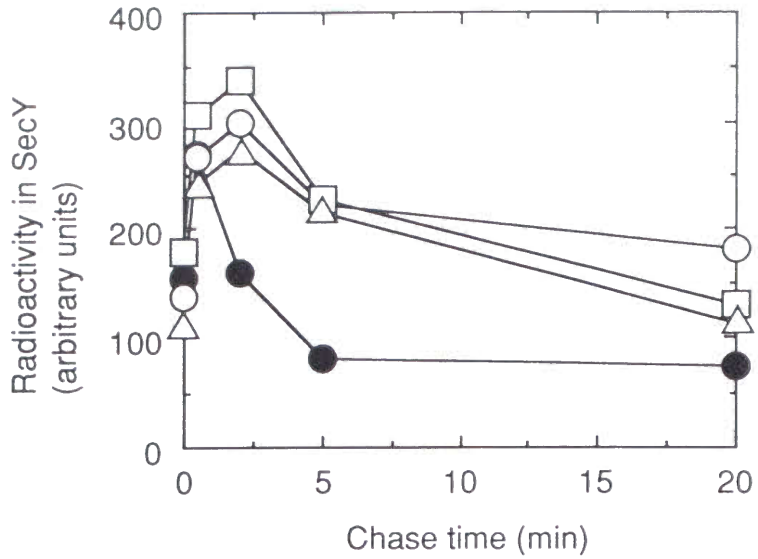


Fig. 8 Degradation of overproduced SecY in *ftsH* cold-sensitive mutants. Cells of AK519 (wild-type)/pKY248 (p*lac-secY*) (closed circles), AK521 (*ftsJ-101*::IS*10*L) /pKY248 (open circles), AK646 (*ftsH102*)/pKY248 (open triangles), and AK525 (*zgj-525*::IS*1*A)/pKY248 (open squares) were grown at 37°C and shifted to 30°C 1 hr before pulse-labeling. Plasmid-encoded *secY* was induced for 10 min, and cells were pulse-labeled with [^{35}S]methionine for 0.5 min followed by chase for 0, 0.5, 2, 5, and 20 min. Radioactive SecY was immunoprecipitated, subjected to SDS-PAGE, and visualized. Radioactivities associated with SecY were quantitated.

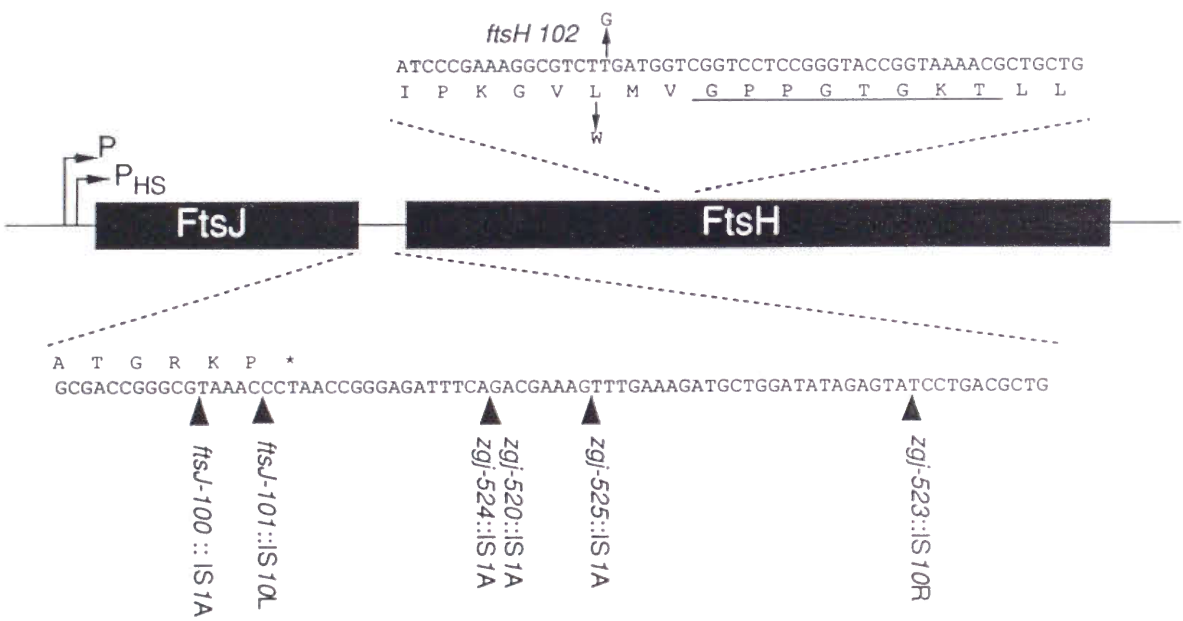


Fig. 9 The positions of IS insertions and a missense mutation that stabilize SecY. The mutations were isolated by the selection/screening procedures described in the text. *ftsH102* is a missense mutation (Leu^{189} to Trp) located closely to one of the ATP binding motifs (GXXXXGKT; underline) of FtsH . Other mutations are insertions of either IS*1*A or IS*10*, which are 768 bp and 1329 bp-long, respectively (Halling *et al.*, 1982; Umeda and Ohtsubo, 1991). The sites of insertions (the upstream ends indicated by the previously defined nucleotide numbers; Tomoyasu *et al.*, 1993) were: 757 for *ftsJ-100*::IS*1*A, 762 for *ftsJ-101*::IS*10*L, 780 for *zgj-520*::IS*1*A, 780 for *zgj-524*::IS*1*A, 788 for *zgj-525*::IS*1*A, and 813 for *zgj-523*::IS*10*R. P and PHS indicate the promoters assigned by Herman *et al.* (1995).

column with open column). At 20°C, the synthesis level of FtsH in wild-type cells was about 60% of the value at 37°C (compare the closed column with the open column). The insertion mutations lowered the expression levels of FtsH at all temperatures examined. The mutational effect was especially pronounced at 20°C (Fig. 10). The low level of FtsH synthesis at low temperature seems to cause the cold-sensitive growth of these mutants.

5. *ftsH* mutants are sensitized to overproduction of SecY

The results presented above indicate that excess and uncomplexed SecY will accumulate in the *ftsH* mutant cells. We then examined whether such accumulation of SecY is deleterious to cell growth or protein export. When SecY was induced from pKY318 in AK520 (*zgj-520*::IS*1*A) cells, the cells grew significantly slower (Fig. 11, open circles) than those of AK519 (*ftsH*$^+$)/pKY318 (closed circles) or AK520 bearing the vector plasmid (open squares).

After induction of SecY, protein export was examined by pulse-chase experiments. Export of Bla, as assessed from kinetics of the appearance of the mature form, was significantly retarded in *zgj-520*::IS*1*A mutant cells (AK520) when they had pKY318 (Fig. 12B, open circles) as compared when they had the vector plasmid (pKY225) (open squares). SecY overproduction had only marginal effect on Bla secretion in the *ftsH*$^+$ cells (compare closed circles with closed squares). Slight retardation in export of MBP was also observed in AK520/pKY318 (Fig. 12A, open circles). These results indicate that accumulation of uncomplexed SecY poses growth disadvantage and causes suboptimal protein export efficiency. These results indicate that elimination of excess SecY molecules will be important for the integrity of the protein export machinery.

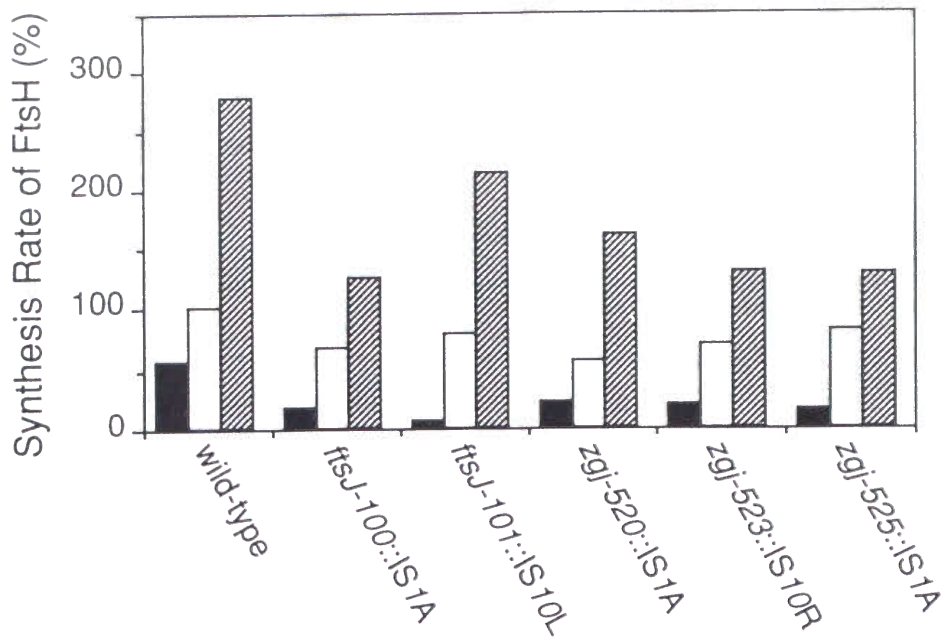


Fig. 10 Lowered expression of FtsH caused by the upstream IS insertion mutations. Cells grown at 37°C to an early log phase were divided into three portions. One was kept at 37°C (open column), whereas others were shifted to 42°C for 5 min (hatched column), or shifted to 20°C for 1 hr (closed column). Cells were pulse-labeled with [^{35}S]methionine for 2 min and chased for 2 min. Equal amounts of radioactive total proteins were used for immunoprecipitation of FtsH, which was separated by SDS-PAGE and quantitated. Values relative to that of the wild-type cells at 37°C are graphically depicted.

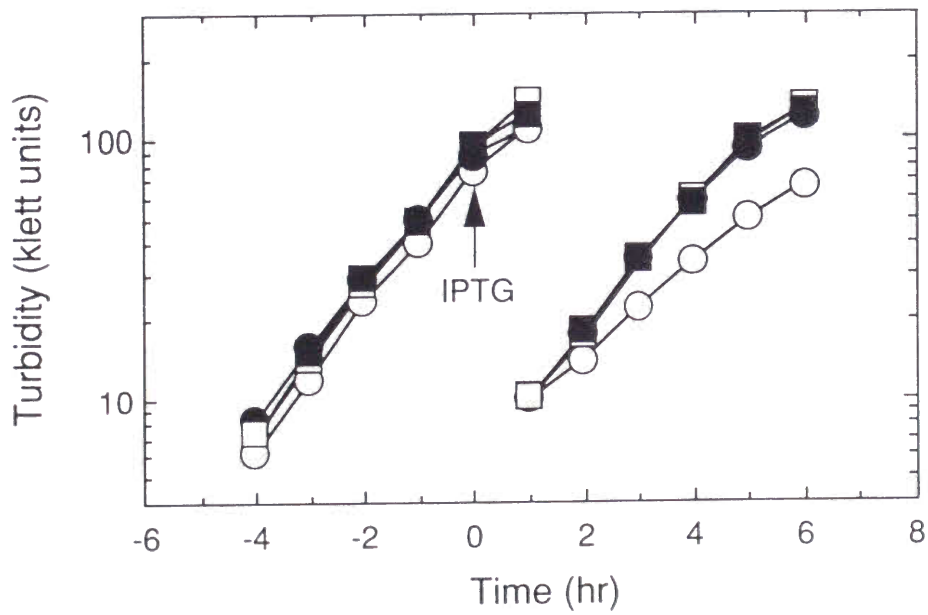


Fig. 11 Effects of SecY overexpression on cell growth.
Cells of AK519 (wild-type)/pKY318 (p*lac-secY*) (closed circles), AK519/pKY225 (vector) (closed squares), AK520 (*zgj-520*::IS*1*A)/pKY318 (open circles) and AK520/pKY225 (open squares) were grown in amino acids-supplemented M9-glycerol medium and induced with 1 mM IPTG as indicated. Turbidity was followed by a Klett colorimeter using a filter #54. The gaps indicate dilutions with prewarmed same medium.

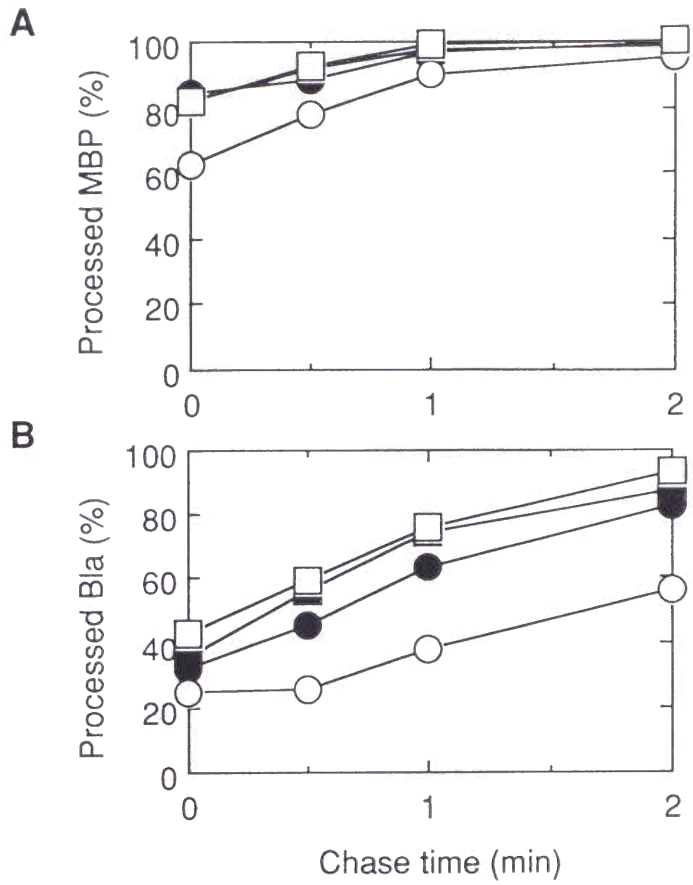


Fig. 12 Effects of SecY overexpression on protein export.
Cells of AK519 (wild-type)/pKY318 (p*lac-secY*) (closed circles), AK519/pKY225 (vector) (closed squares), AK520 (*zgj-520*::IS*1*A)/pKY318 (open circles) and AK520/pKY225 (open squares) were grown in amino acids-supplemented M9-glycerol medium in the presence of 1 mM IPTG and 0.2% maltose for 3 hr. They were pulse-labeled with [^{35}S]methionine for 0.5 min, and chased with unlabeled methionine for 0, 0.5, 1 and 2 min. MBP (**A**) and Bla (**B**) were immunoprecipitated. Values represent proportions of the mature forms.

II. Interaction between HflKC and FtsH and its functional significance

1. Mutations in *hflK* and *hflC* that stabilize uncomplexed SecY

As shown above, we isolated a number of mutants defective in the degradation of SecY, and all of such mutations proved to affect either expression level or primary structure of *ftsH* . In the mutant isolation described above, we chose cold-sensitive mutants that appeared at a high frequency for characterization. To identify additional genes involved in degradation of SecY, we repeated the screening, but without the preference given to cold-sensitive clones. From 10 independent bacterial cultures, 20 mutants were saved. Transduction mapping showed that 15 of them were probably *ftsH* mutants. The remaining 5 mutants, unlinked to *ftsH*, were characterized. We obtained Tn*5* insertions that were co-transducible with one of these mutations and cloned the chromosomal regions around the insertions using the kanamycin-resistance marker. Chromosomal localization of the insertions were examined by hybridization of the clones with the filter-immobilized *E. coli* genomic DNA library (Kohara *et al.*, 1987). One of the Tn*5* insertions named *zjf-803*::Tn*5* was found to be located at the 95 min region of the chromosome, where *hflA* had been mapped. When a plasmid carrying the *hflA* region (pKH142) was introduced into the mutant linked with *zjf-803*::Tn*5*, the SecY-LacZα protein was destabilized again (Fig. 13). We subcloned each of the *hflX*, *hflK* and *hflC* genes under the *lac* promoter control (Fig. 13). Plasmid pKH145 carrying *hflK*$^+$ complemented the mutation (see Fig. 15, column 14) that was then referred to as *hflK13*. Two of the remaining 4 mutants also proved to have mutations in the *hflA* region. One (named *hflK11*) was complemented by pKH145 and the other (named *hflC9*) was complemented by pKH146 (*hflC*$^+$) (see Fig. 15, column 10). The remaining two mutations were mapped at the 22 min region. They had an internal deletion mutation in the *yccA* open reading frame (Tamura *et al.*, 1984) as described below in detail.

We cloned *hflK11*, *hflK13* and *hflC9* alleles and determined their nucleotide

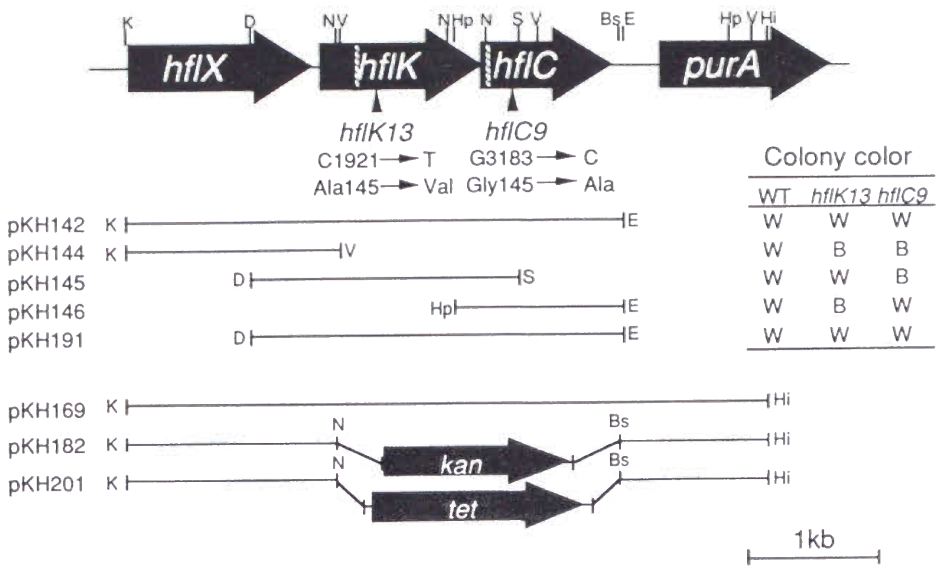


Fig. 13 The *hflA* class of mutants that are defective in degradation of excess SecY and SecY24 mutant protein.
Using the selection/screening procedures described in the text, we isolated SecY-stabilizing mutations (*hflK13* and *hflC9*) in the *hflA* locus of the chromosome. This figure depicts the schematic representation of the *hflA-purA* region of the chromosome. Thick arrows at the top represent the open reading frames (hatched regions represent the putative transmembrane regions; Noble *et al.*, 1993). The mutation sites at the levels of nucleotide and predicted amino acid sequences, and plasmids carrying various parts of this region are also shown. The nucleotide numbering is according to Noble *et al.* (1993). Note that all the chromosomal segment cloned into the plasmids were placed under the control of the *lac* promoter. The arrows with *kan* and *tet* notations represent the segments that substituted for the chromosomal segments. W and B on the right represent colony color of white and blue, respectively, for the wild-type (WT, strain AK861), *hflK13* (AK865) and *hflC9* (AK863) strains that carried pKY258 (*secY-lacZ*α) as well as one of the *hflA* region plasmids shown on the left. Thus, "W" signifies that overproduced SecY was unstable, whereas "B" signifies that overproduced SecY was stabilized. Abbreviations for the restriction sites are: K, *Kpn*I; D, *Dra*I; N, *Nru*I; V, *Eco*RV; Hp, *Hpa*I; S, *Sal*I; Bs, *Bsu*36I; E, *Eco*RI; Hi, *Hin*dIII.

alterations. It was found that *hflK11* and *hflK13* had the same base substitution, C^{1921} to T, in *hflK*, resulting in an Ala^{145} to Val change in the HflK protein. Probably, these mutants represented siblings, since they originated form the same initial culture, and we saved the nomenclature of *hflK13* for them. The *hflC9* allele contained a single G^{3183} to C base substitution in *hflC*, which corresponds to Gly^{145} to Ala change in the protein product.

2. Mode of involvement of the *hflK* and *hflC* gene functions in SecY degradation

Pulse-chase and immunoprecipitation experiments showed that half-life of overproduced SecY in the *hflK13* (Fig. 14A, closed squares) and *hflC9* (closed circles) mutant cells was about 5 min whereas that in the wild-type cells was about 2 min (open circles). Thus, these missense mutations stabilized excess SecY. However, SecY was not stabilized in a mutant in which a chromosomal segment encompassing the *hflK* and *hflC* genes had been replaced by a kanamycin resistance determinant (data not shown). These results indicate that the HflK-HflC protein complex is not positively required for the proteolysis of SecY and that *hflK13* and *hflC9* were not loss-of-function mutations. In agreement with this notion, overexpression of these mutant genes from plasmids in wild-type cells resulted in accumulation of the overproduced SecY in the cell (Fig. 15, columns 4 and 5). Pulse-chase and immunoprecipitation experiments showed that SecY was significantly stabilized when either HflK13 (Fig. 14B, closed squares) or HflC9 (closed circles) was overproduced together with the respective wild-type partner. Simultaneous overproduction of HflK13 and HflC9 caused more marked stabilization of SecY than overproduction of either one of them (closed triangles). Thus, these mutant proteins inhibited the degradation of SecY in additive manners.

When FtsH was overproduced from a plasmid in the *hflK13* or *hflC9* mutant cells, SecY degradation was restored (Fig. 15, columns 8 and 13), suggesting that HflK13 and HflC9 antagonized the FtsH action. Since it is difficult to envision that the

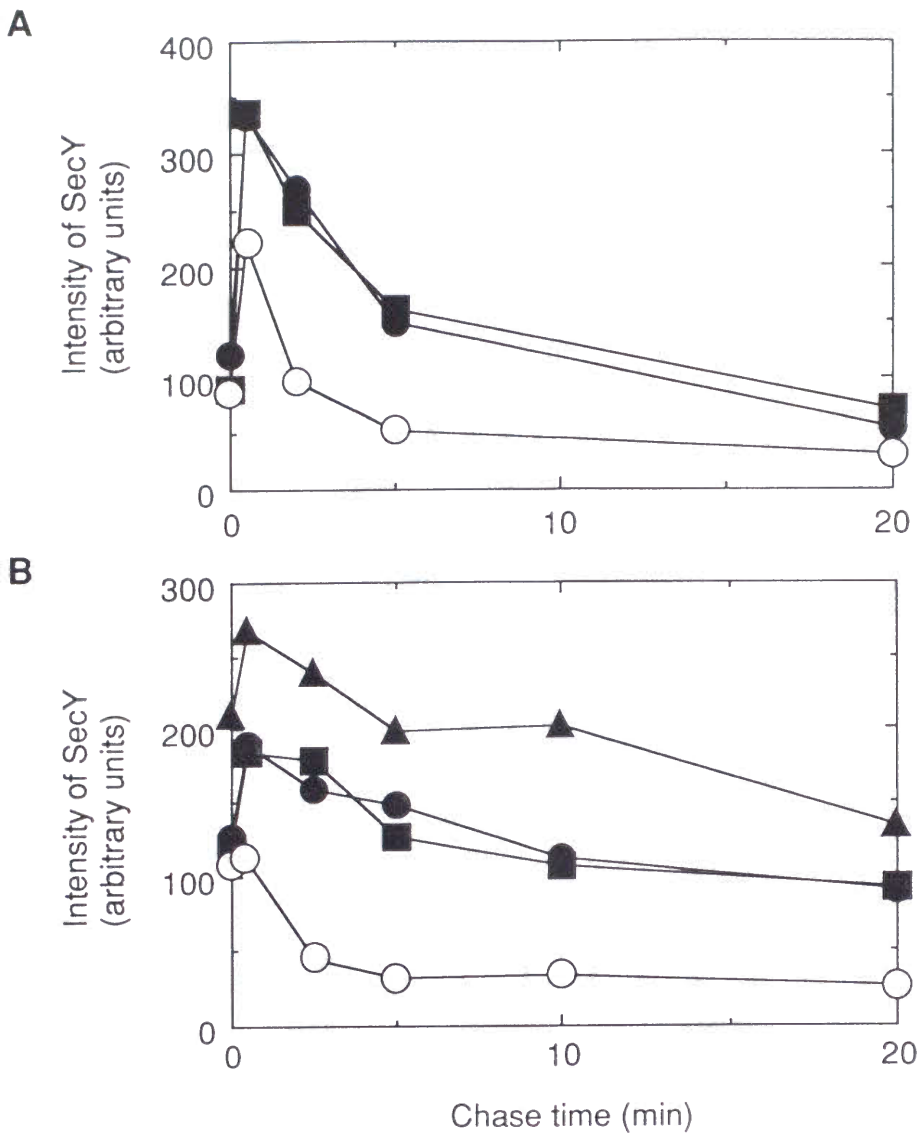


Fig. 14 Effects of the *hflC9* and *hflK13* mutations on stability of oversynthesized SecY. **A**. Cells of AK861 (wild-type)/pKY248 (p*lac-secY*) (open circles), AK863 (*hflC9*)/pKY248 (closed circles), and AK865 (*hflK13*)/pKY248 (closed squares) were grown at 37°C. Plasmid-encoded *secY* was induced for 10 min, and cells were pulse-labeled with [^{35}S] methionine for 20 sec followed by chase for indicated periods. **B**. Cells of AD16/pKY248/pKH191 (p*lac-hflK*$^{+}$*hflC*$^{+}$) (open circles), AD16/pKY248/pKH193 (p*lac-hflK*$^{+}$*hflC9*) (closed circles), AD16/pKY248/pKH194 (p*lac-hflK13hflC*$^{+}$) (closed squares), and AD16/pKY248/pKH290 (p*lac-hflK13hflC9*) (closed triangles) were grown at 37°C, and *secY* and *hflK-hflC* were induced for 1hr. Cells were pulse-labeled with [^{35}S]methionine for 30 sec, and chased for indicated periods, followed by immunoprecipitation of SecY and SDS-PAGE. Radioactivities associated with SecY were quantitated.

occurrence of the gain-of-function *hflK13* and *hflC9* mutations was fortuitous, these results seem to suggest that even the wild-type products of the *hflK/hflC* genes interact with FtsH. The experimental results shown below reveal that this is indeed the case.

3. FtsH and HflKC in the membrane are crosslinkable

Possible interaction of FtsH with HflKC was examined by chemical crosslinking. Membranes were prepared from the wild-type (AD16) and *ΔhflK-hflC* (AK990) cells that had been pulse-labeled with [^{35}S]methionine, and treated with a cleavable and membrane-permeable crosslinker, DSP. Proteins were then solubilized with SDS and immunoprecipitated using anti-FtsH or anti-HflKC antibodies. Immunoprecipitates were analyzed by SDS-PAGE, following reductive cleavage of the crosslinks (Fig. 16; see also Fig. 31 and Fig. 33). Anti-FtsH brought down the bands designated FtsH$^{\#}$, HflK, HflK′ and HflC from the wild-type sample that was treated with DSP (Fig. 16, lane 1). Without crosslinking, only FtsH was seen (lane 2). Neither HflK, HflK′ nor HflC was detected even after crosslinking when the *ΔhflK-hflC::kan* strain was used (lane 3). FtsH$^{\#}$ might have been produced by the covalent attachment of the DSP moieties to FtsH. The identities of HflK and HflC were confirmed by recovery of these proteins by second immunoprecipitation with anti-HflKC antibodies (data not shown). The band HflK' was a proteolytic fragment of HflK (Cheng *et al.*, 1988), and it was probably generated by contaminating outer membrane protease, OmpT, since it was not observed when *ompT*-disrupted cells were used (see Figs. 31 and 33).

Anti-HflKC brought down FtsH$^{\#}$ as well as HflK, HflK′ and HflC (Fig. 16, lane 5). Without crosslinking, only the latter three proteins were recovered (lane 6). No specific protein was recovered from membranes from the deletion strain (lanes 7 and 8). Again, the identity of the FtsH$^{\#}$ band was confirmed by second immunoprecipitation (data not shown). These experiments demonstrate that HflK and HflC are the nearest

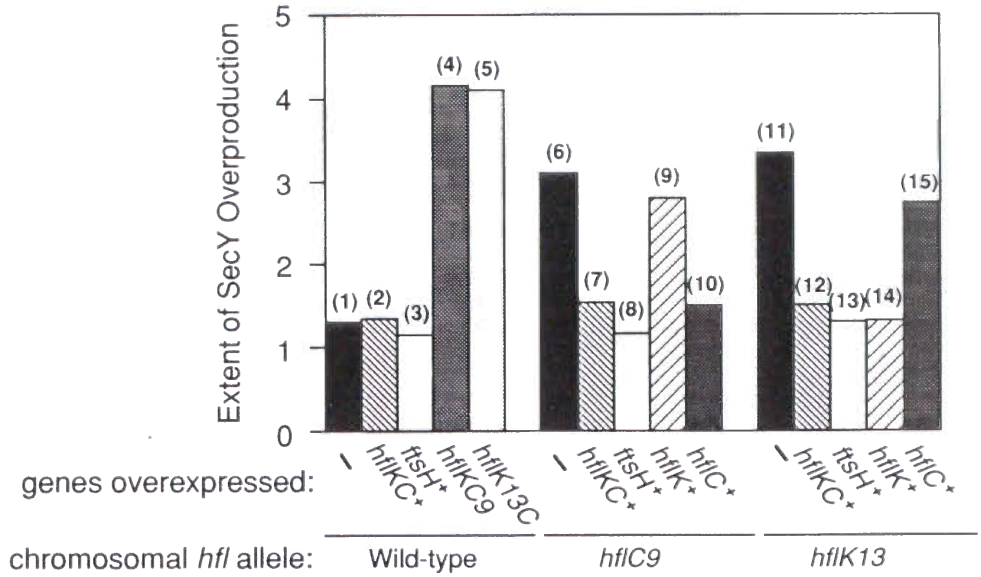


Fig. 15 Accumulation of overproduced SecY in cells with different *hflA/hflB* configurations. Strains AK861 (*hflK*$^+$ *hflC*$^+$), AK863 (*hflK*$^+$ *hflC9*), and AK865 (*hflK13 hflC*$^+$) were transformed with two plasmids. One was pKY248 which overexpresses SecY about 4-5 fold the chromosomal level (Taura *et al.*, 1993). Another plasmid that each strain carried is shown at the bottom of each column; pMW119 was a vector, pKH191 overexpressed *hflK*$^+$-*hflC*$^+$, pKH198 overexpressed *ftsH*$^+$, pKH193 overexpressed *hflK*$^+$ and *hflC9*, pKH194 overexpressed *hflK13* and *hflC*$^+$, pKH145 overexpressed *hflK*$^+$, and pKH146 overexpressed *hflC*$^+$. Cells were grown at 37°C in M9 medium supplemented with 18 amino acids (20 μg/ml, other than Met and Cys), thiamine (2 μg/ml), 0.4% glycerol and appropriate antibiotics and induced with 1 mM IPTG for 3 hr. Whole-cell proteins (2 μg and 4 μg) were separated by SDS-PAGE, for detection of SecY by immunoblotting. Densitometric quantitation of the SecY intensities was done by a Discovery Series Densitometer (PDI). The intensity of the SecY in wild-type cells (AK861) without any plasmid was taken as unity, and relative values are depicted.

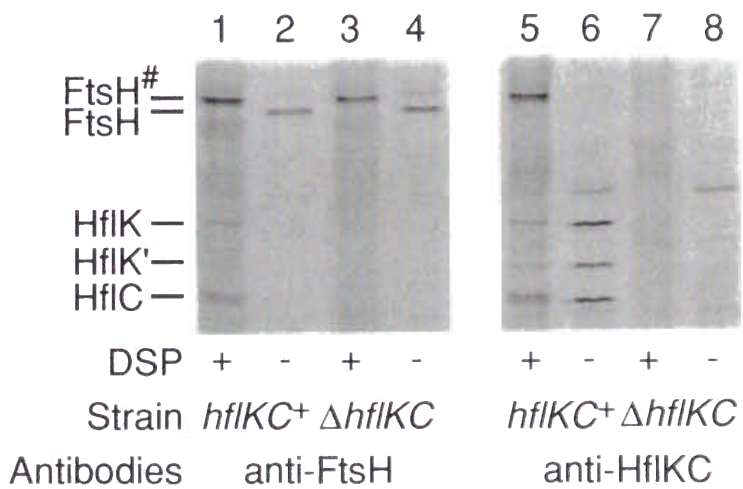


Fig. 16 Crosslinking of FtsH and HflKC.
Cells of AD16 (*ftsH*$^+$; lanes 1, 2, 5, and 6) and AK990 (Δ*hflK-hflC*::*kan*; lanes 3, 4, 7, and 8) were labeled at 37°C with [^{35}S]methionine for 10 min. Total membrane fractions were treated with DSP (lanes 1, 3, 5, and 7) or its solvent, DMSO (lanes 2, 4, 6, and 8). Membrane proteins were solubilized with SDS and subjected to immunoprecipitation using anti-FtsH antibodies (lanes 1 to 4) or anti-HflKC (lanes 5 to 8). Immunoprecipitates were solubilized in SDS sample buffer containing 2-mercaptoethanol, separated by SDS-PAGE, and visualized by autoradiography. HflK' indicates an artificial breakdown product of HflK, and FtsH$^{\#}$ indicates a DSP-modified form of FtsH.

neighbors of FtsH and vice versa.

4. FtsH and HflKC remain complexed after membrane solubilization

To demonstrate FtsH-HflKC interaction more directly, we performed immunoprecipitation experiments under non-denaturing conditions. Membranes were prepared from pulse-labeled cells, solubilized with a non-ionic detergent, NP40, and subjected to immunoprecipitation with anti-FtsH. As expected, FtsH was the major protein in the immunoprecipitates (Fig. 17, lane 1). In addition, numerous radioactive bands were observed. To discriminate between bands of non-specific backgrounds and those specifically brought down by the antibodies, anti-FtsH was preincubated with an excess of antigenic peptide. Now five major bands (HflKC and asterisks in lane 1) disappeared whereas the other bands remained unchanged, indicating that the former bands were specific (lane 2). Two of the specific bands coincided with HflK and HflC in the electrophoretic mobilities and they were indeed precipitated with anti-HflKC when the first immunoprecipitates were denatured and subjected to the second immunoprecipitation (lane 3). The recovery of a small amount of FtsH in the second immunoprecipitates (lane 3) was probably due to some renaturation of the FtsH antibodies under the experimental conditions used. HflK and HflC, once denatured, did not themselves react with the anti-FtsH antibodies (data not shown). We conclude that HflK and HflC in the solubilized membrane extract were co-immunoprecipitated with FtsH. In the above experiments, 3 additional polypeptides (62 kDa, 60 kDa, and 31 kDa; lane 1, asterisks) were specifically coimmunoprecipitated with FtsH.

Interaction between FtsH and HflKC was demonstrated by an independent method. We constructed a mutant in which the chromosomal *ftsH* was replaced by *tet* and *ftsH-his$_6$-myc*, a fusion gene that encoded FtsH with attached C-terminal His$_6$ and Myc-tag sequences (FtsH-His$_6$-Myc) (Akiyama *et al.*, 1995). When NP40-solubilized membrane proteins from this mutant (AK1181) were subjected to affinity enrichment

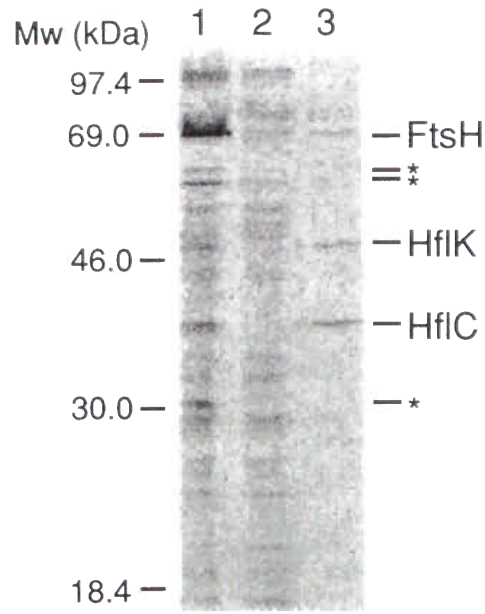


Fig. 17 Co-immunoprecipitaion of FtsH and HflKC.
Cells of AD202 were labeled with [^{35}S]methionine for 10 min. Membrane proteins were solubilized under non-denaturing conditions and precipitated with affinity-purified anti-FtsH antibodies in the absence (lanes 1) or presence (lane 2) of an excess of the FtsH epitope peptide. Immunoprecipitates obtained in a reaction identical to that shown in lane 1 were dissociated with SDS and subjected to the second immunoprecipitation with anti-HflKC serum (lane 3). An autoradiogram after separation by SDS-PAGE is shown. Proteins indicated by asterisks were specific since they were competed out by the antigen peptide.

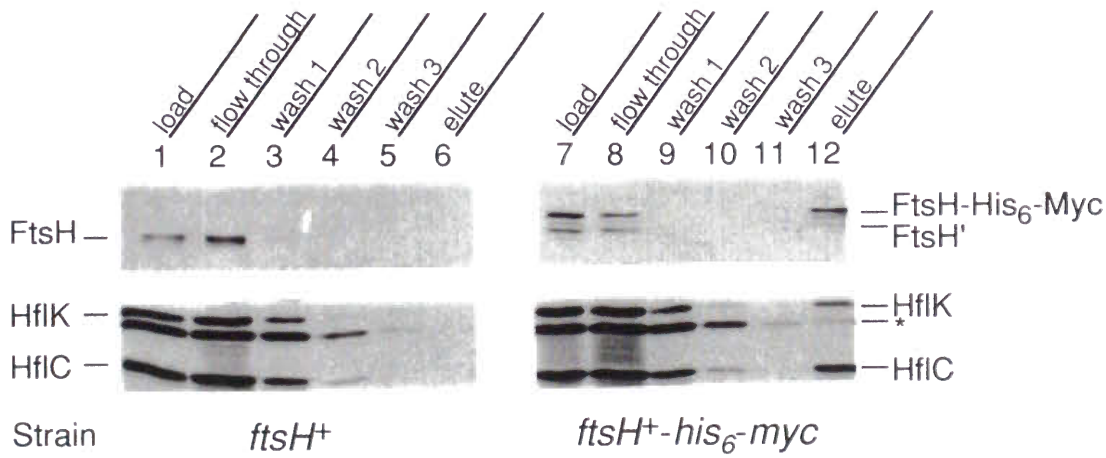


Fig. 18 Co-elution of HflKC with FtsH-His$_6$-Myc in Ni-NTA-agarose affinity chromatography. Membranes prepared from AD202 (*ftsH*$^+$; lanes 1-6) and AK1181 (*ftsH*$^+$-*his*$_6$-*myc*; lanes 7-12) were solubilized and subjected to Ni-NTA-agarose chromatography (load, lanes 1 and 7; flow-through, lanes 2 and 8). Proteins adsorbed to Ni-NTA-agarose were washed three times (lanes 3-5 and 9-11) and eluted with 250 mM imidazole (lanes 6 and 12). Proteins in each fraction were separated by SDS-PAGE and visualized by immunoblotting using antisera against FtsH (upper part) or HflKC (lower part). The asterisk indicates a nonspecific background. FtsH' indicates the C-terminally-cleaved product of FtsH-His$_6$-Myc.

using Ni-NTA-agarose, not only FtsH-His_6-Myc but also HflK and HflC were eluted with 250 mM imidazole (Fig. 18, lane 12). Without FtsH-His_6-Myc, no HflKC was recovered in the imidazole fraction (lanes 1-6). A minor band designated as FtsH' (lane 7-12) probably represented a C-terminally cleaved product of FtsH-His_6-Myc (Akiyama *et al.*, 1995); its recovery in the imidazole eluate should have been due to the homo-oligomeric interaction between the FtsH polypeptides (Akiyama *et al.*, 1995). Both the immunoprecipitation and the histidine-tagging experiments demonstrated that FtsH interacted with HflKC with an affinity high enough to keep the complex after solubilization of membranes with non-ionic detergent.

To obtain information about molecular size of the FtsH-HflKC complex, solubilized membrane proteins were fractionated by a gel filtration column. We found that purified FtsH tends to oligomerize or aggregate when incubated at 30-37℃ in the absence of ATP, which prevented the oligomerization/aggregation (Akiyama *et al.*, submitted). When NP40-solubilized membrane extract was fractionated by a Superose 6 column, FtsH was mainly eluted at the position of about 400 kDa, whereas HflK and HflC, which always co-fractionate, were eluted at a position around 200 kDa (data not shown). Co-elution of FtsH and HflKC was not very clear under these conditions, partly because of the broad distributions of these membrane proteins and low resolution of the column. When a membrane preparation that was treated by apyrase to remove residual ATP was incubated at 30℃ for 30 min and then solubilized, a major fraction of FtsH was now eluted at a higher molecular mass region (about 2,000 kDa), whereas a minor fraction was eluted at around 600 kDa (Fig. 19A, open circles). A fraction of HflKC was also eluted at the latter position, whereas a major peak of HflKC was at about 200 kDa position (Fig. 19B, open circles). Interestingly, inclusion of ATP-Mg^{2+} during the incubation of membrane resulted in increased 600 kDa peaks of FtsH and HflKC (Fig. 19, closed circles) with concomitant decrease in the 2,000 kDa FtsH peak and the 200 kDa HflKC peak. These results suggest that association between FtsH and HflKC is modulated by ATP, which stimulates the

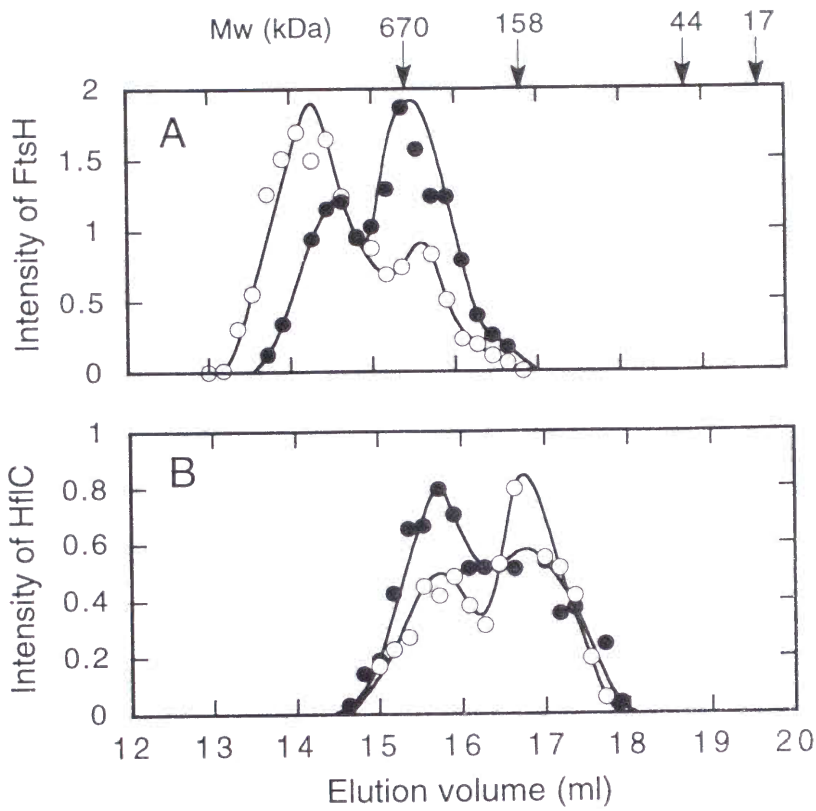


Fig. 19 Gel-filtration profiles of FtsH and HflKC in NP40-solubilized membrane samples. Membranes prepared from AD202 were incubated with (closed circles) or without (open circles) 5 mM $MgCl_2$-5 mM ATP at 30°C for 30 min and then solubilized with NP40. Fractions of Superose 6 size exclusion chromatography were subjected to SDS-PAGE and immunoblotting with anti-FtsH (**A**) or anti-HflKC (**B**) antibodies. FtsH (**A**) and HflC (**B**) were quantitated by a Discovery Series Densitometer (PDI). The markers used in gel filtration were: thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), and equine myoglobin (17 kDa).

complex formation. The complex, once formed, remains in the NP40-solubilized states. The 2,000 kDa species of FtsH, which was produced upon incubation of the membrane extract in the absence of ATP, probably represented non-physiological aggregates of FtsH.

5. Functional significance of the FtsH-HflKC interaction

The *secY24* mutational alteration in the fourth cytoplasmic domain of SecY impairs SecY-SecE interaction (Baba *et al.*, 1994). As a result, the SecY24 protein is slowly degraded at 42°C, leading to an insufficient intracellular amount of SecY. Mutations in *ftsH* can suppress the temperature-sensitivity of the *secY24* mutant, since they stabilize the mutant SecY protein (Fig. 4-6). The *ΔhflK-hflC* mutation did not itself affect cell growth. However, when it was combined with the *secY24* mutation, the resulting double mutant (*secY24 ΔhflK-hflC*: strain AK1194) grew poorly even at 30°C. The *secY24* mutant cells form abnormal, ghost-like colonies on rich medium after prolonged (~2 days) incubation at 42°C (Shiba *et al.*, 1984). The *secY24 ΔhflK-hflC* double mutant cells showed this phenotype much earlier (~10 hr), and HflKC overproduction reversed this effect. The synthetic growth defect with *ΔhflK-hflC* was specific for the *secY24* mutation, since the *secY39* (Cs) mutation (Baba *et al.*, 1990) did not show such a property. The IS insertion mutation, *zgj-525*::IS*1*A, which lowers the expression level of *ftsH*, partially suppressed the synthetic growth defect when introduced into the *secY24 ΔhflK-hflC* strain; the resulting triple mutant strain (AK1244) again required 2 days incubation at 42°C to exhibit the abnormal colony morphology. Thus, the FtsH functions appear to be required for the exacerbated growth defect caused by the combination of *ΔhflK-hflC* and *secY24*.

Effects of the *ΔhflK-hflC* mutation on stability of the SecY24 protein was examined by pulse-chase and immunoprecipitation experiments. At 30°C, degradation of SecY24 was moderate (Baba *et al.*, 1994), and no apparent degradation was observed up to 20 min after initiation of chase (Fig. 20A, closed circles). In the *ΔhflK-hflC* background,

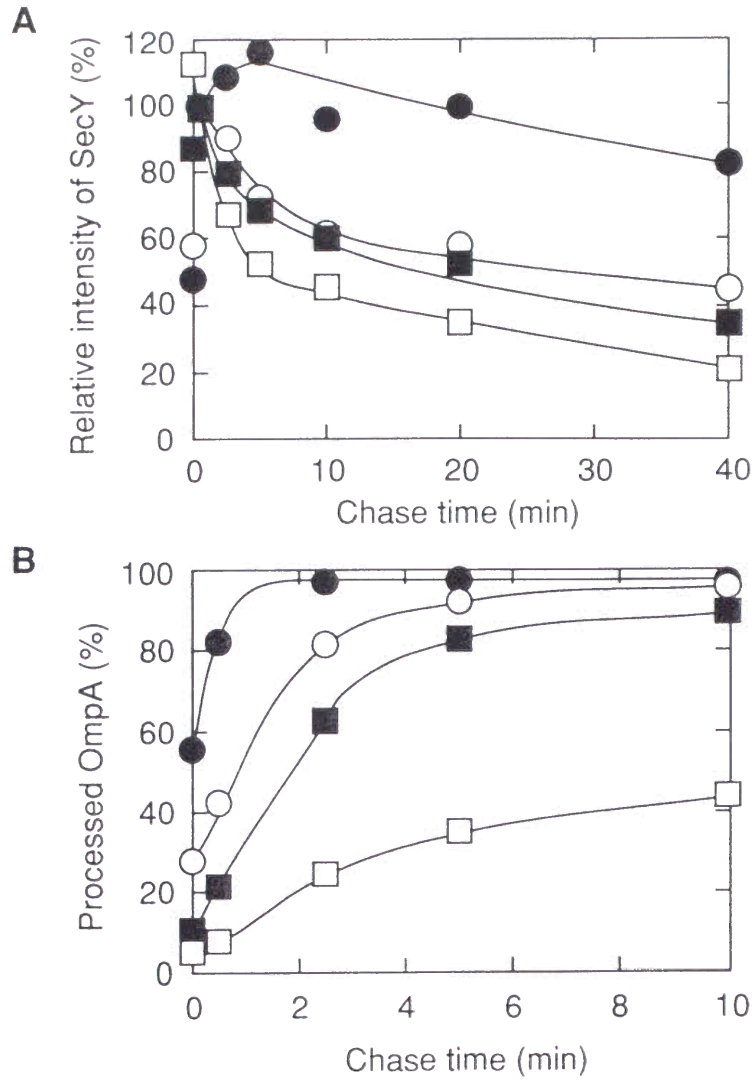


Fig. 20 Effects of a *hflK-hflC* deletion on phenotypes of the *secY24* mutant. Cells of AK1191 (*secY24*; circles) and AK1194 (*secY24 ΔhflK-hflC*; squares) were pulse-labeled with [^{35}S]methionine for 0.5 min and chased with unlabeled methionine for the indicated periods at 30°C (closed symbols) or at 42°C (1 hr after a 30 to 42°C temperature shift) (open symbols). SecY (**A**) and OmpA (**B**) were immunoprecipitated and visualized by autoradiography after SDS-PAGE. About 7.8×10^5 cpm of total cell proteins were used in each immunoprecipitation. Values relative to that of 0.5 min chase are shown in (**A**), whereas proportions of the mature form in OmpA are shown in (**B**).

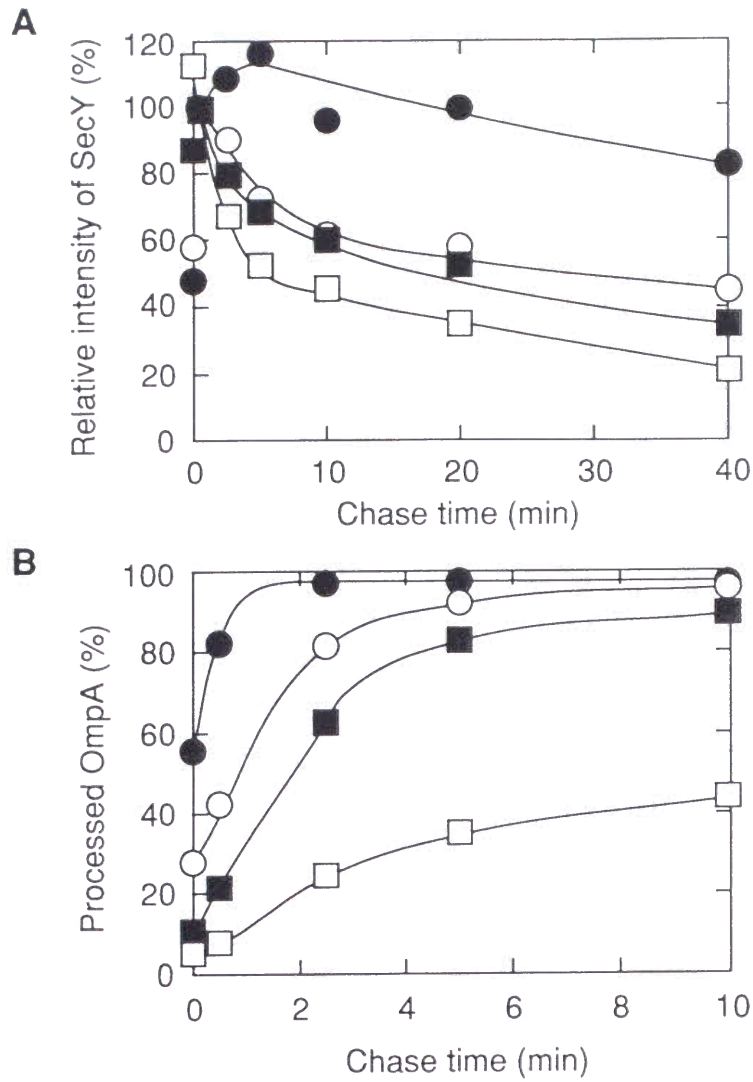


Fig. 20 Effects of a *hflK-hflC* deletion on phenotypes of the *secY24* mutant. Cells of AK1191 (*secY24*; circles) and AK1194 (*secY24 ΔhflK-hflC*; squares) were pulse-labeled with [^{35}S]methionine for 0.5 min and chased with unlabeled methionine for the indicated periods at 30°C (closed symbols) or at 42°C (1 hr after a 30 to 42°C temperature shift) (open symbols). SecY (**A**) and OmpA (**B**) were immunoprecipitated and visualized by autoradiography after SDS-PAGE. About 7.8×10^5 cpm of total cell proteins were used in each immunoprecipitation. Values relative to that of 0.5 min chase are shown in (**A**), whereas proportions of the mature form in OmpA are shown in (**B**).

40% of newly synthesized SecY24 was degraded in 10 min (closed squares), indicating that it was destabilized significantly even at 30°C. At 42°C, SecY24 was naturally unstable, but its instability was increased in the Δ*hflK-hflC* background (compare open circles and open squares). Export kinetics of OmpA was examined by measuring the export-dependent signal sequence processing. Export in the *secY24* mutant cell was found to be slower in the presence of the Δ*hflK-hflC* mutation than in the presence of the wild-type alleles of these genes. This was true both at 30°C (Fig. 20B, compare closedcircles and closed squares) and at 42°C (compare open circles and open squares). Although it was difficult to compare degradation rates of overproduced SecY in the presence and absence of the Δ*hflK-hflC* mutation, since degradation was too rapid in both cases, we believe it very likely that wild-type HflKC protein acts negatively with respect to the FtsH-mediated proteolysis of SecY.

To directly investigate the inhibitory action of HflKC against the SecY-degrading activity of FtsH *in vitro*, we overproduced the HflK/HflC complex and purified it. Membranes from HflKC-overproducing cells were solubilized with NP40 (Fig. 21A, lane 5), and fractionated by a series of chromatographies (lanes 6-8). The final preparation was apparently free of contaminations. We also purified FtsH-His_6-Myc by Ni-NTA agarose affinity chromatography followed by gel filtration chromatography (Fig. 21B), using a Δ*hflK-hflC* cells (AK1272) bearing the *ftsH-his$_6$-myc* plasmid (pSTD113). The final preparation of FtsH-His_6-Myc (lane 7) contained a product (FtsH') of proteolytic cleavage around the junction between FtsH and His_6-Myc tag (Akiyama *et al.*, 1995). Taking this into account, the purity was about 90%. SecY was degraded when it was incubated with FtsH-His_6-Myc at 37°C in the presence of ATP (Fig. 22, lanes 1-3).

The HflKC preparation did not itself degrade SecY to a measurable extent (Fig. 22, lanes 7-9). When FtsH-His_6-Myc preparation was preincubated with HflKC, degradation of SecY was inhibited (lanes 4-6). Treatment of the HflKC preparation at 75°C did not affect the solubility of this protein in the detergent solution, but it

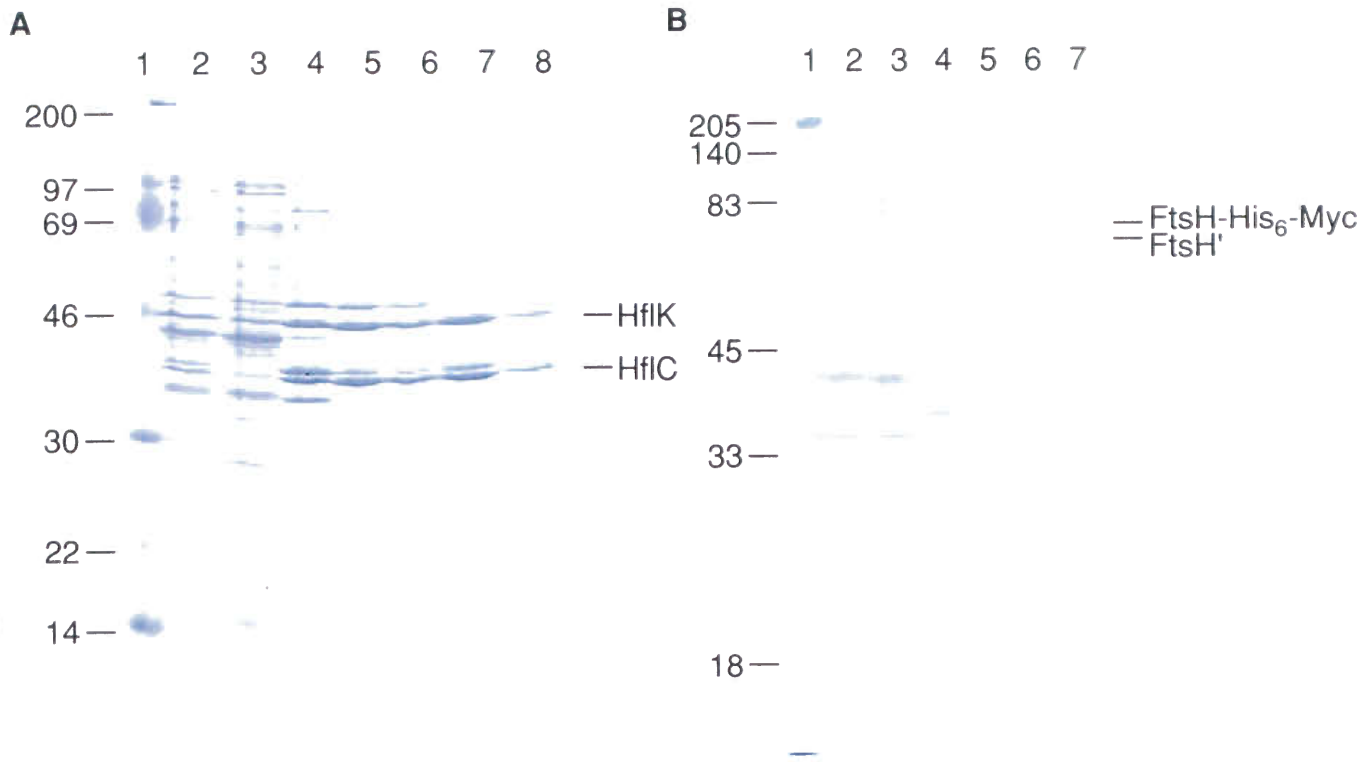


Fig. 21 Purification of HflKC and FtsH-His_6-Myc.
Protein profiles during purification of HflKC (**A**) and FtsH-His_6-Myc (**B**) are shown as coomassie brilliant blue-stained SDS-PAGE patterns. (**A**) Lane 1, molecular weight markers; lane 2, total cellular proteins; lane 3, soluble fraction; lane 4, crude membrane fraction; lane 5, NP40-solubilized membrane fraction; lane 6, DEAE-Sepharose fraction; lane 7, Superdex 200 fraction; lane 8, hydroxyapatite fraction. (**B**) Lane 1, molecular weight markers; lane 2, total cellular proteins; lane 3, soluble fraction; lane 4 crude membrane fraction; lane 5, NP40 -solubilized membrane fraction; lane 6, Ni-NTA-agarose fraction; lane 7 Superose 6 fraction.

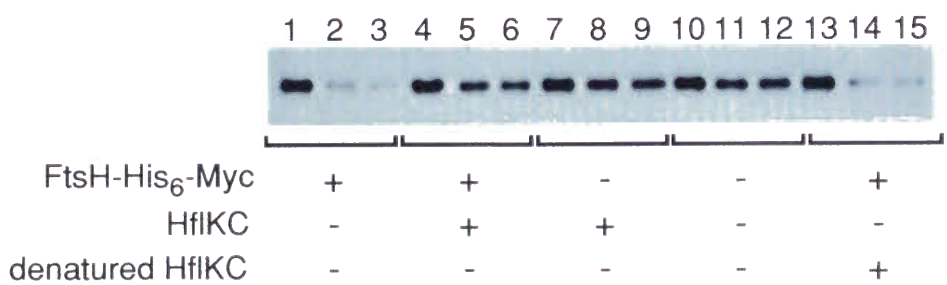


Fig. 22 *In vitro* effects of HflKC on FtsH-mediated degradation of SecY.
The following samples were preincubated at 4°C for 1 hr: lanes 1-3, FtsH-His_6-Myc alone; lanes 4-6, FtsH-His_6-Myc and HflKC; lanes 7-9, HflKC alone; lanes 10-12, buffer alone; lanes 13-15, HflKC that had been denatured by incubation at 75°C for 15 min and FtsH-His_6-Myc. Purified SecY was added to each sample and incubated at 37°C for 0 (lanes 1, 4, 7, 10, and 13), 0.5 (lanes 2, 5, 8, 11, and 14), and 1 (lanes 3, 6, 9, 12, and 15) hr. Reactions were terminated by adding SDS sample buffer, and SecY was visualized by immunoblotting following SDS-PAGE.

abolished the ability of HflKC to inhibit the FtsH-mediated degradation of SecY (lanes 13-15). Thus, it is likely that HflKC acts as an inhibitor of SecY degradation and heat denaturation inactivates the inhibitory action of HflKC. Taken together with the *in vivo* results, we propose that HflKC is a negative modulator of the SecY-degradation activity of FtsH.

6. *In vitro* proteolytic activities of FtsH and HflKC against the λ CII protein

Although HflKC has been reported to be a protease which degrade λ CII protein independent of FtsH (HflB) (Cheng *et al.*, 1988; Banuett *et al.*, 1986; Herman *et al.*, 1993), we demonstrated that HflKC and FtsH form a complex. We also showed that FtsH has a protease activity against the SecY protein whereas HflKC inhibits FtsH. Therefore, we examined whether these properties of FtsH and HflKC can be extended to the proteolysis of λ CII protein *in vitro*. For this examination we first isolated partially purified radio-labeled CII protein, after its overproduction and labeling with [^{35}S]methionine. When the CII preparation was mixed with purified FtsH-His$_6$-Myc and incubated at 37°C in the presence of ATP, it was degraded (Fig. 23A, lanes 1-3). This proteolytic reaction required hydrolysis of ATP since a non-hydrolyzable analog, ATPγS, did not substitute for ATP (lanes 4-6). Omission of ATP and inclusion of apyrase also inhibited the reaction (lanes 7-9). The reaction was time dependent, occurring slightly faster at 37°C (Fig. 23B, lanes 7-12) than at 30°C (lanes 1-6). No appreciable degradation of CII occurred without FtsH-His$_6$-Myc (data not shown). Recently, Shotland *et al.* (1997) reached the same conclusion that FtsH degrades CII *in vitro*.

Our preparation of purified HflKC protein did not significantly degrade CII under several different buffer conditions (data not shown). When FtsH was preincubated with HflKC, the CII degradation activity of FtsH was abolished (Fig. 23C). During this preincubation, degradation of neither FtsH-His$_6$-Myc nor HflKC occurred (data not shown). These results raise a serious question about the validity

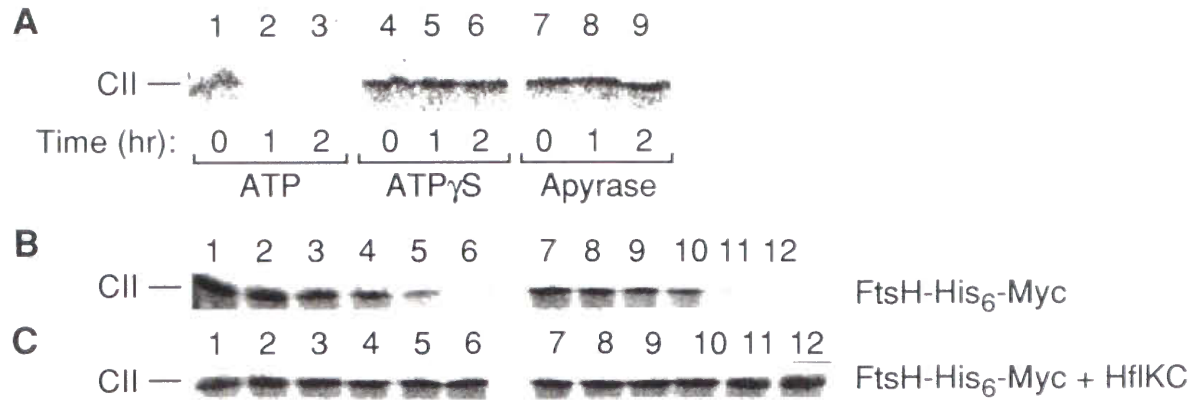


Fig. 23 FtsH degrades CII and HflKC antagonizes the degradation *in vitro*.
A. Purified FtsH-His_6-Myc (5 μg) and [^{35}S]methionine-labeled CII (about 15,000 cpm) were incubated at 37°C in the presence of 5 mM ATP (lanes 1 to 3), 5 mM ATPγS (lanes 4 to 6), or 50 units/ml apyrase (lanes 7 to 9) for 0 (lanes 1, 4, and 7), 1 (lanes 2, 5, and 8), and 2 (lanes 3, 6, and 9) hr. **B**. and **C** . Purified FtsH-His_6-Myc (0.8 μg) alone (**B**) or FtsH-His_6-Myc (0.8 μg) and HflKC (3.2 μg) (**C**) were preincubated at 0°C for 1 hr. [^{35}S] methionine labeled CII (about 10,000 cpm) was then added to each sample and incubated for 0 (lanes 1 and 7), 5 (lanes 2 and 8), 10 (lanes 3 and 9), 20 (lanes 4 and 10), 40 (lanes 5 and 11) and 80 (lanes 6 and 12) min at 30°C (lanes 1-6) or at 37°C (lanes 7-12). After SDS-PAGE, CII was visualized by autoradiography.

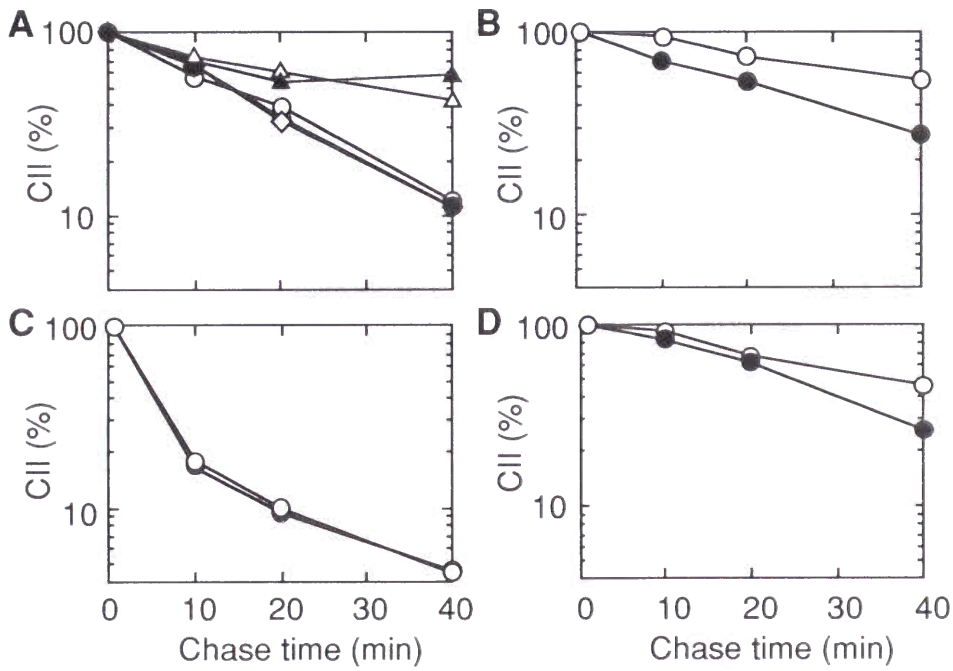


Fig. 24 Stability of the λ CII protein under various conditions *in vivo*.
Cells of AD16 (*hflK*$^+$ *hflC*$^+$; closed circles), AK1339 (*hflC150*; open circles), AK990 (*ΔhflK-hflC*; open diamonds), AK525 (*zgj-525*::IS*1*A; closed triangles), and AK1301 (*ΔhflK-hflC zgj-525*:: IS*1*A; open triangles) were transformed with pKH274 (*para-cII*), induced for the ara transcription, and subjected to pulse-chase analysis under the temperature conditions specified below. Pulse-labeling with [^{35}S]methionine was for 0.5 min and chase was conducted for 0.5, 10, 20, and 40 min. After SDS-PAGE separation, radioactivities associated with CII were quantitated. Values relative to that at 0.5 min chase point are shown for each curve. **A**, cells were grown at 37°C throughout; **B**, cells were grown first at 30°C, shifted to 43°C for 4 min, pulse-labeled at this temperature, and chased at 30°C; **C**, cells were treated as in (B) except that chase was done at 43°C; **D**, Cells were grown at 30°C throughout.

of the proposal that HflKC is a protease acting against CII (Cheng *et al.*, 1988; Hoyt *et al.*, 1982).

7. *In vivo* roles of FtsH and HflKC in degradation of λ CII protein

Hoyt *et al.* (1982) as well as Banuett *et al.* (1986) examined stability of λ CII protein that was expressed from the *cII* gene cloned under the control of the λ p*L* promoter. Their results show that overproduced CII was stabilized both in the *hflA150* and *hflA1* mutants as well as in the *hflB29* mutant (*hflB29* is an allele of *ftsH* ; Herman *et al.*, 1993). We carried out similar experiments using a plasmid with *cII* cloned under the *ara* promoter control. In accordance with the results of Banuett *et al.* (1986) we observed that CII was markedly stabilized in the *zgj-525*::IS*1*A mutant in which the expression level of FtsH was decreased (Fig. 24A, compare closed triangles and closed circles). However, we failed to observe any appreciable differences between the wild-type cells (closed circles) and *ΔhflK-hflC* cells (open diamonds) with respect to the stability of the CII protein. Similarly, *ΔhflK-hflC* did not affect the CII stability when combined with the *zgj-525*::IS*1*A mutation (compare open triangles with closed triangles). Thus, the absence of HflKC affects neither the rapid CII degradation in the presence of normal FtsH function nor the slower CII degradation in cells of insufficient FtsH supply. The kinetics of degradation of CII in the *hflA150* mutant cells (open circles) were also identical to those in the wild-type cells (closed circles). Our sequencing results showed that the *hflA150* mutation is a Mu phage insertion (Hoyt *et al.*, 1982) between T^{3450} and A^{3451} residues of the *hflA* operon (nucleotide numbering, according to Nobel *et al.* (1993)) within the *hflC* gene.

Although the experiments described above were carried out with cells grown at 37°C, Hoyt *et al.* (1982) as well as Banuett *et al.* (1986) grew cells first at 31°C, shifted them to 43°C to inactivate the CI857 repressor, followed by pulse-labeling at this temperature and a chase at 31°C. When we exactly followed this temperature-shift scheme, CII was indeed more stable in the *hflA150* mutant (Fig. 24B, open circles)

than in the wild-type strain (closed circles). Note that degradation of CII at 30°C was slower than that observed at the 37°C. When chase was done at 43°C, CII was degraded more rapidly than at 37°C (compare Fig. 24C and Fig. 24A), and we failed to observe any difference between *hflA150* cells and the wild-type cells (Fig. 24C, compare open and closed circles). When cells were grown at 30°C throughout, CII was slightly more stable in the *hflA150* mutant (Fig. 22D, open circles) than in the wild-type strain (closed circles). Essentially the same effects of temperature were observed when the *ΔhflK-hflC* cells were examined (data not shown). The possibility that the intrinsically rapid degradation at the high temperature masked the *ΔhflK-hflC* effect can be ruled out, since *ΔhflK-hflC* did not affect still slower degradation observed in the *zgj-525*::IS*1*A mutant cells (Fig. 24A). These results indicate that the stabilization of CII by the *hflA* mutations was specific for the particular growth temperature (30-31°C) and, even at this temperature, the effects were only moderate. In contrast, stabilization of CII by the *ftsH*-lowering *zgj-525*::IS*1*A mutation was much clearer and observed at 37°C, the temperature where lysogenization frequency of λ is usually measured. The *ΔhflK-hflC* mutation itself did not affect the synthesis levels of FtsH (data not shown).

To examine the roles of FtsH and HflKC in the stability control of CII under more physiological conditions, UV-irradiated cells were infected with λ, and CII was pulse-labeled and chased. Under these conditions, CII in wild-type cells was degraded with a half-life of about 1.5 min at 37°C (Fig. 25A, closed circles). It was stabilized to half lives of about 5 min both in the *zgj-525*::*IS*1A (closed triangles) and in the *ΔhflK-hflC* cells (closed squares). Stabilization of CII in λ-infected *hflA* mutant cells was also reported previously (Hoyt *et al.*, 1982). At 30°C where *zgj-525*::IS*1*A mutant had severer cellular defects, the stabilization of CII in this mutant was enhanced (Fig. 25B; closed triangles). On the other hand, stability of CII in the *ΔhflK-hflC* cells at 30°C was not significantly different from that at 37°C (closed squares). Curiously, however, when HflKC was overproduced in wild-type cells, CII was stabilized to a

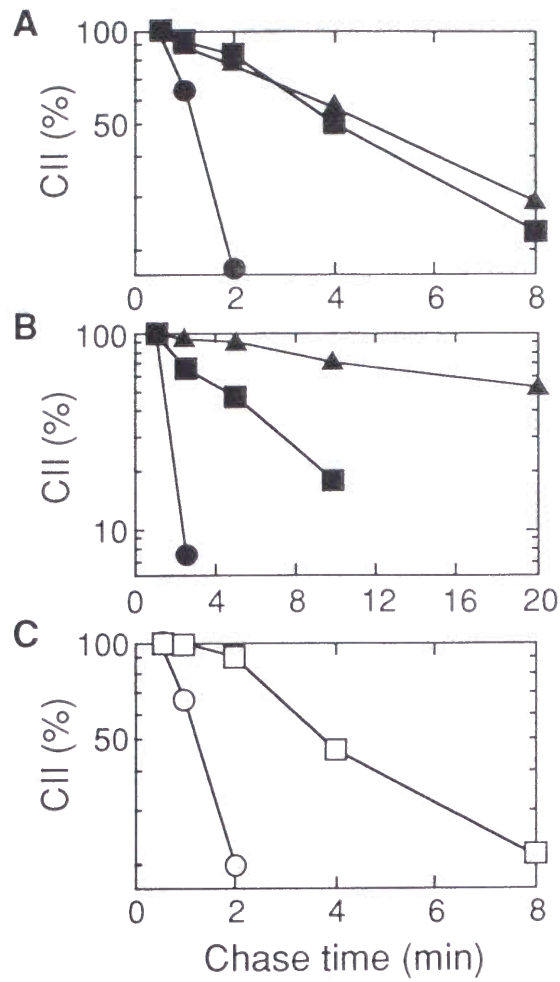


Fig. 25 Stability of CII in λ-infected cells.
Cells of AD16 (wild-type; closed circles), AK990 (*ΔhflK-hflC*; closed squares), AK525 (*zgj-525*::IS*1*A; closed triangles), AD16/pBAD18 (vector; open circles), and AD16/pKH178 (p*ara-hflKC*; open squares) were grown at 37°C (**A**, **C**) or at 30°C (**B**) to a mid-log phase. To the latter 2 strains, 0.4% arabinose was added 1 hr before UV-irradiation. Cells were UV-irradiated and infected with λ. After 5 min (37°C) or 8 min (30°C), cells were pulse-labeled for 1 min with [^{35}S]-methionine and chased as indicated. Total labeled proteins were precipitated by TCA and separated by SDS-PAGE. Radioactivities associated with the CII band were quantitated, and values relative to those without chase are presented.

similar extent as observed in the *ΔhflK-hflC* strain (Fig. 25C, open squares). When an FtsH overproducer was infected with λ, CII was hardly detected even at the 0.5 min chase point, due presumably to an accelerated degradation. These results are fully consistent with FtsH being the CII degrading protease. However, the HflA effects were more complicated, since both of its absence and overproduction gave similar CII-stabilizing effects.

Table I Effects of *hfl* mutations on lysogenization of λ

Exp.	Strain	Relevant genotype	Plasmid carried	Lysogenization frequency (%)	λc17 propagation†
1	AD16	WT	–	2.4	+
2	AK990	*ΔhflKC*	–	40.6	–
3	AK863	*hflC9*	–	1.7	+
4	AK865	*hflK13*	–	1.5	+
5	AK525	*zgj-525*::IS1A	–	81.9	–
6	AD16	WT	pBAD18 (vector)	3.0	+
7	AD16	WT	pKH178 (*hflK*$^+$*C*$^+$)	42.8	–
8	AK1127	*hflC*::*tet*	pMW119 (vector)	31.3	–
9	AK1127	*hflC*::*tet*	pKH146 (*hflC*$^+$)	4.5	+
10	AK1127	*hflC*::*tet*	pKH339 (*hflCΔ165-200*)	6.7	+

Cells were pregrown either at 30°C (for Exp. 1-5) or at 37°C (for Exp. 6-10), but lysogenization frequency were measured all at 37°C. See Materials and Methods for further details about the media and other growth conditions before infection.

† Cells were cross-streaked with λc17 on TB agar and cell lysis/plaque formation was scored after overnight incubation at 37°C. This phage cannot propagate in cells in which lysogenization if preferred (Banuett *et al.*, 1986; Herman *et al.*, 1993).

8. Paradoxical effects of *hflKC* mutations and HflKC overexpression on the lysogenic decision

We measured λ lysogenization frequencies using different host strains. As reported previously by Herman *et al.* (1993), the *ΔhflK-hflC* mutation increased the lysogenization

frequency from a few percent in wild-type to about 40% (Table I, Exp. 2). The *zgj-525*::IS*1*A mutation (pre-grown at 30°C) with a decreased FtsH content did so much more pronouncedly (about 80% lysogenization; Exp. 5). Interestingly, the HflKC-overproducing plasmid, in contrast to the empty vector, also increased the lysogenization to a similar extent as the *hflK-hflC* deletion (Exp. 7). These results agree well with the CII stability data obtained in the λ-infected cells. The *hflC9* and the *hflK13* mutations, which were identified as SecY-stabilizing and partially dominant mutations in this study, did not significantly increase the λ lysogenization frequency (Exp. 3 and 4). All the results of lysogenization were confirmed by λc17 growth tests (Table I) (Banuett *et al.*, 1986; Herman *et al.*, 1993). The c17 mutation results in the constitutive synthesis of CII (Packman and Sly, 1968; Rosenberg *et al.*, 1978), stabilization of which by an *hfl*⁻ results in overaccumulation of CII and inhibition of the lytic growth of λc17. λc17 formed clear plaques on wild-type, *hflC9*, and *hflK13* cells (Exp. 1, 3, and 4), but could not grow on Δ*hflK-hflC* cells or *zgj-525*::IS*1*A cells (Exp. 2 and 5). Wild-type cells (AD16) harboring HflK-HflC overproducing plasmid (pKH178) did not allow the lytic growth of λc17 (Exp. 7), while those harboring vector plasmid (pBAD18) did so (Exp. 6).

9. The proposed serine protease active site region can be deleted from HflC without major dysfunction

It was noted that *E. coli* HflC has a ClpP-like sequence motif (Fig. 26A and B, blue) and that Ser^{197} (Fig. 26, red) residue could serve as a serine protease active site (Noble *et al.*, 1993). Recently, genes for homologs of HflK and HflC were discovered in *Haemophilus influenzae* (Fleischmann *et al.*, 1995) and *Vibrio parahaemolyticus* (McCarter, 1994). Although the HflC homologs in *H. influenzae* and *V. parahaemolyticus* show high overall sequence similarities to the *E. coli* counterpart (*H. influenzae*, 58% identity and 82% similarity; *V. parahaemolyticus*, 55% identity and 80% similarity), the ClpP-like domain is not conserved (Fig. 26B). Most notably, *H. influenzae* HflC lacks

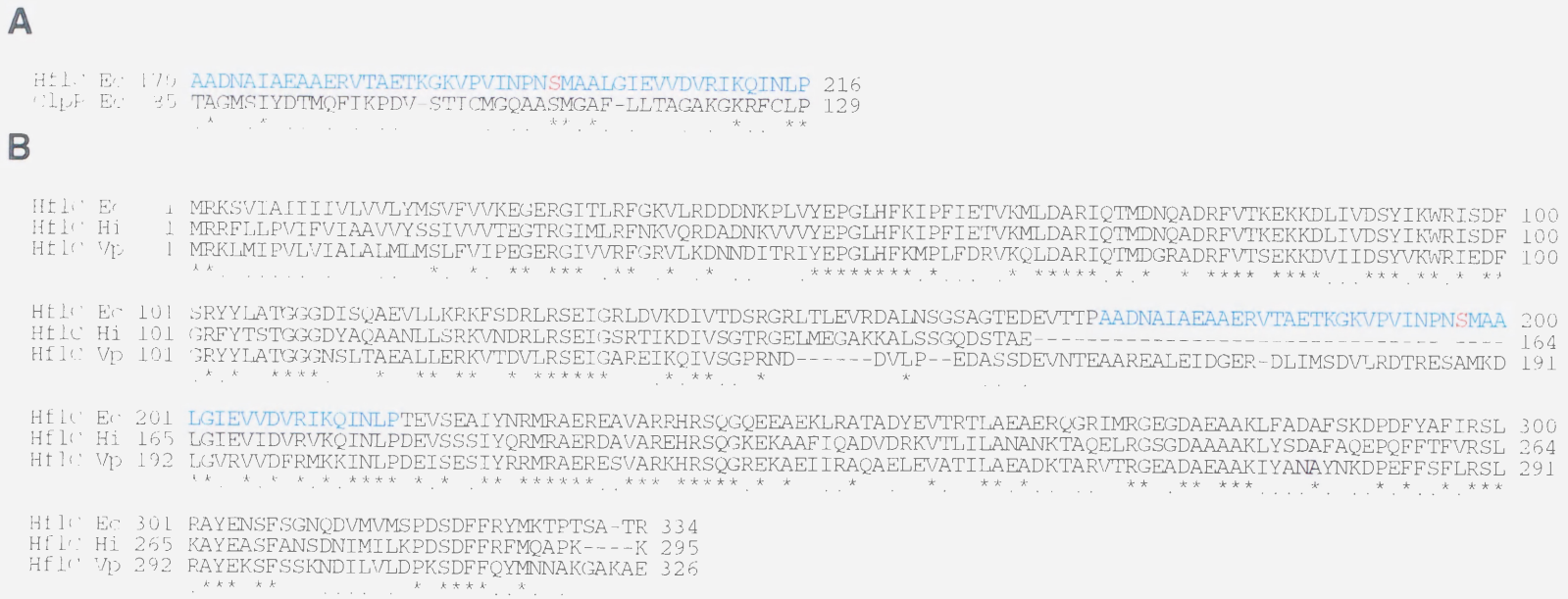


Fig. 26 The ClpP-like domain found in *E. coli* HflC is not conserved among its homologs in other bacteria. **(A)** Homology alignment of *E. coli* HflC protein with the *E. coli* ClpP protease, a serine protease, assigned by Noble *et al.* (1993). Putative active site serine residue (Ser-197) is coloured with red. **(B)** Homology alignment of *E. coli* HflC, *Haemophilus influenzae* HflC, and *Vibrio parahaemolyticus* HflC. HflC proteins in these bacteria were highly conserved: *H. influenzae* HflC is 58 % identical and 82 % similar to *E. coli* HflC, *V. parahaemolyticus* HflC is 55 % identical and 80 % similar to *E. coli* HflC. However, the ClpP-like domain (blue) found in *E. coli* HflC is not conserved. *H. influenzae* HflC lacks a 36 residue segment that overlaps the ClpP-like domian. In *V. parahaemolyticus* HflC, the residue corresponding to *E. coli* Ser-197 is Ala.

a 36 residue segment that overlaps the ClpP-like domain (Fig. 26B). We deleted this 36 amino acid segment (Glu-165 to Ala-200) from HflC by constructing the *hflCΔ165-200* mutation on a plasmid (pKH339). This plasmid was able to lower the λ lysogenization frequency observed in the *hflC::tet* strain (AK1127) from 31% (with the empty vector (pMW119)) to 6.7% (Table I, Exp. 10). The *hflC*$^+$ plasmid (pKH146) gave a lysogenization frequency of 4.5%. The λc17 phage cannot propagate on *hflC::tet* strain harboring the vector but can grow on that harboring pKH339 (*hflCΔ165-200*). Thus, HflC with the *Δ165-200* internal deletion retains the ability to maintain λ losogenization frequency to a low level. The ClpP-like domain in HflC is dispensable for its function as a lysogenization controller.

10. The major domains of HflK and HflC are periplasmically oriented

Both the HflK protein and the HflC protein contain, at their N-terminal regions, a hydrophobic stretch that can traverse the membrane. However, their orientations have not been determined experimentally. The protease hypothesis implicitly assumes that the C-terminally located main hydrophilic domains of these proteins are located in the cytoplasmic side, since CII is a cytosolic protein. We directly examined orientations of the HflK and the HflC proteins by protease digestion experiments. When intact spheroplasts were treated with proteinase K, both of HflK (45.5 kDa) and HflC (37.6 kDa) disappeared (Fig. 27A, lane 2). Since GroEL, an internal control for a cytosolic protein, remained undigested unless Triton X-100 was added (Fig. 27A, bottom panel), it was suggested that major domain of HflK and HflC are located externally. When inverted membrane vesicles, prepared by passing the spheroplasts through a French pressure cell, were treated with proteinase K, a membrane-protected 40 kDa band (HflK* in Fig. 27B) was produced that reacted with anti-HflK (Fig. 27B, lower panel). This is consistent with a digestion of a part of the N-terminal hydrophilic segment (79 residues) from the cytoplasmic side. The HflC band was almost completely protected from digestion in the inverted membrane vesicles, consistent with the

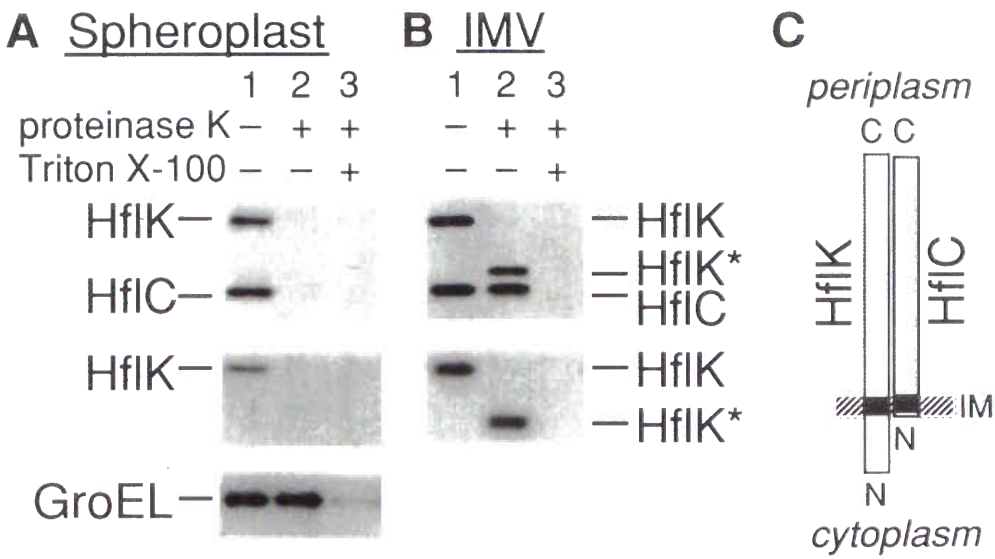


Fig. 27 HflK and HflC reside on the periplasmic side of the membrane. Spheroplasts (**A**) and inverted membrane vesicles (**B**) prepared from AD202 were incubated at 0°C for 2 hr in the absence (lane 1) or presence (lanes 2 and 3) of 1 mg/ml proteinase K. Lanes 3 received 1% Triton X-100 before the digestion. Proteins were analyzed by SDS-PAGE and immunoblotting, using antisera directed against the HflKC complex (upper panel), the HflK subunit (medium (A) or lower (B) panel), and the GroEL protein (bottom panel in A). **C**, a schematic representation of the topographical arrangements of HflK and HflC.

presence of only three amino acids that are located N-terminally to the transmembrane segment. Thus, the HflC protein, the suspected protease subunit, contains virtually no cytosolic residues. The fact that the large hydrophilic domain of each of these proteins resides in the periplasmic space indicate that HflKC exerts transmembrane modulation over the proteolytic activity of FtsH.

III. Identification of a new membrane protein substrate, YccA, of FtsH and use of its mutant forms to study substrate specificity of FtsH

1. A SecY-stabilizing mutation in *yccA*

We characterized a remaining SecY-stabilizing mutant described above. We first obtained a Tn*5* insertion (*zcc-554*::Tn*5*) that was co-transducible with the mutation at a frequency of about 90% (Fig. 28, arrowhead) in P1 transduction. A chromosomal segment including this Tn*5* was cloned (pKH28) and a fragment (Fig. 28, Probe) was shown to hybridize with clones 9B10 and 4H9 of the *E. coli* genomic DNA library (Kohara *et al.*, 1987). Thus, the Tn*5* insertion was located between *helD* and *hyaA* at the 22 min region of the chromosome (Fig. 28). Results of P1 transduction suggested the following order of markers: *pyrD*/*zcc-554*::Tn*5*/SecY-stabilizing mutation (Fig. 28). We cloned several overlapping chromosomal fragments of this region (Fig. 28), and found two plasmids (pKH125 and pKH93) that slightly decreased the β-galactosidase activity of the mutant cells expressing the SecY-LacZα fusion protein. This weak complementation activity was further localized within a 0.9-kb *Taq*I interval (Fig. 28, pKH135; Table II, Exp. 9). This region contains single open reading frame, *yccA*, of unknown function (GenBank accession number, X00547; Tamura *et al.*, 1984) and we designate the mutation *yccA11*. We cloned the *yccA* region from the mutant chromosome and determined the nucleotide sequence, revealing a 24 bp in-frame deletion within *yccA* and close to the 5' terminus. The mutation corresponds to a deletion of amino acid residues 5-12 of the protein product (Fig. 29A).

The wild-type product of *yccA* is expected to be a polypeptide of 219 amino acids (23.5 kDa), highly enriched in hydrophobic ones. The YccA sequence is consistent with its having seven transmembrane segments (Fig. 29B). The TopPredII 1.1 program (Claros and von Heijne, 1994) predicts that the first, third, fifth, and seventh hydrophilic regions of YccA are on the cytoplasmic side of the membrane. Thus, the *yccA11* deletion is within the amino terminal cytoplasmic region. We constructed a *yccA*-disrupted strain having a *kan* insertion within the chromosomal *yccA* gene (*yccA*::*kan*).

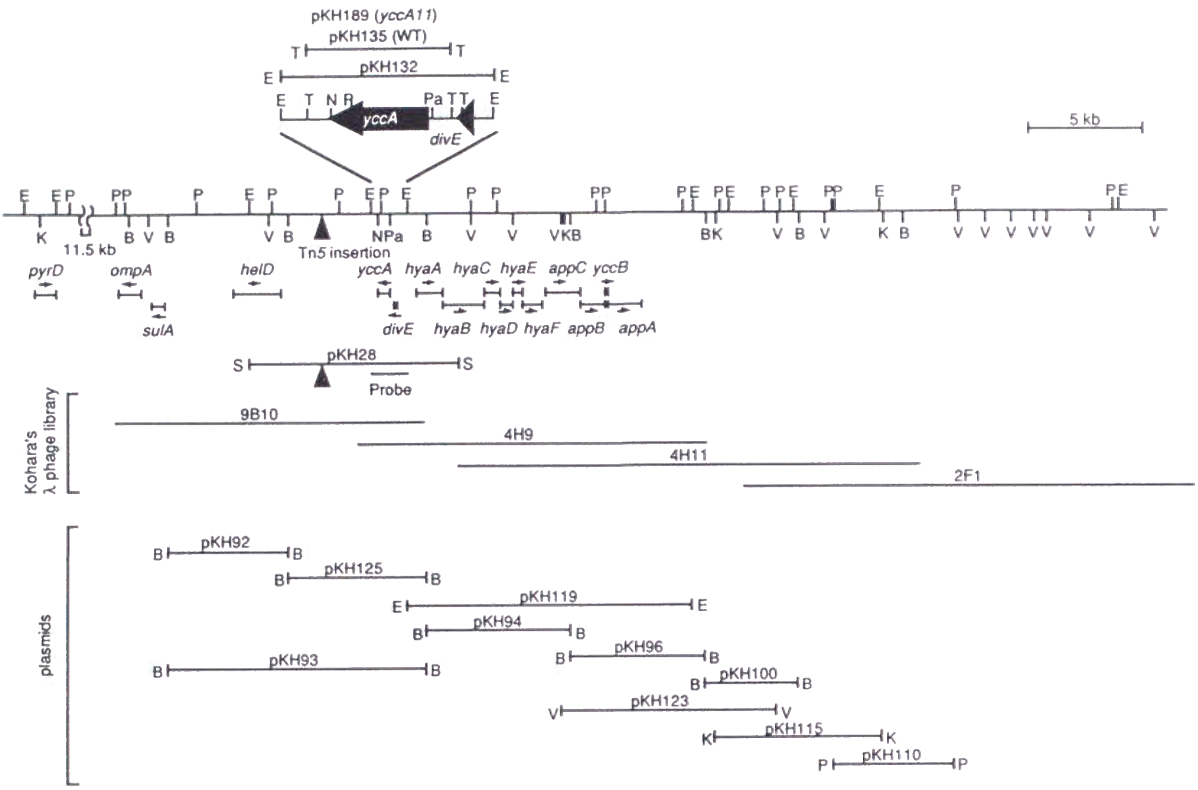


Fig. 28 The 22 min region of the *E. coli* chromosome.
The physical map of the 22 min region of the *E. coli* chromosome is shown. 9B10, 4H9, 4H11, and 2F1 are λ phage clones described by Kohara *et al.* (1987). The vertical arrowhead shows the location of *zcc-554*::Tn*5* obtained in this study. "Probe" indicates the fragment used for hybridization mapping. All the plasmids shown are derivatives of pSC101, and the chromosomal segments cloned are shown by horizontal lines. Abbreviations for restriction enzymes: E, *Eco*RI; B, *Bam*HI; K, *Kpn*I; P, *Pvu*II; S, *Sau*3AI; T, *Taq*I; V, *Eco*RV.

A

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GAATTCATGGTGATGAAATGATCAATCTGCTGTTATTTCACGCTATCAATGCACTTTTGGCTGTTTTTCAGGCAAACAAACAAATTAGGGGTTTACACGC   100
CGCATCGGGATGTTTATAGTGCGCGTCATTCCGGAAGTGTGGCCGAGCGGTTGAAGGCACCGGTCTTGAAAACCGGCGACCCGAAAGGGTTCCAGAGTTC   200
GAATCTCTGCGCTTCCGCCAAATAAGATAAGGGGTTAGCTAAATGCTAACCCCTTTTTCTTTTGCCTGTCGAAATTCTCAGGGCGTTATATTTGCTTAAT   300
GACCTGATAATCCGCTGTTAAACCTGTTCCAGATCAAATGCGTAAAGATGGGTAAAACTTCTGGGTGCCCTTACGCATTATCATTATGCTGCTTAATTAA   400
                                                                                    -35
TTACATCTGTCATAGAGAGTGACTCAATGGATCGTATTGTTAGTTCTTCACATGACCGTACATCACTGCTTAGCACCCATAAGGTGCTGCGTAATACCTA   500
-10          SD      M  D  R  I  V  S  S  S  H  D  R  T  S  L  L  S  T  H  K  V  L  R  N  T  Y          25
                                  yccA11 deletion
TTTTCTGCTGAGCCTGACGCTGGCCTTTTCGGCGATTACCGCAACTGCCAGTACGGTGCTGATGCTGCCATCTCCGGGTCTGATTCTGACGCTGGTGGGT   600
F  L  L  S  L  T  L  A  F  S  A  I  T  A  T  A  S  T  V  L  M  L  P  S  P  G  L  I  L  T  L  V  G    58

ATGTATGGTTTGATGTTCCTGACCTATAAAACGGCGAATAAGCCGACCGGGATTATCTCCGCATTCGCCTTTACCGGTTTTCTGGGTTATATCCTCGGAC   700
M  Y  G  L  M  F  L  T  Y  K  T  A  N  K  P  T  G  I  I  S  A  F  A  F  T  G  F  L  G  Y  I  L  G  P    92

CTATTCTGAACACCTATCTGTCTGCCGGAATGGGTGACGTAATCGCTATGGCACTGGGCGGAACGGCGTTAGTGTTCTTCTGCTGCTCTGCATATGTGCT   800
   I  L  N  T  Y  L  S  A  G  M  G  D  V  I  A  M  A  L  G  G  T  A  L  V  F  F  C  C  S  A  Y  V  L   125

GACCACCCGCAAAGATATGTCGTTCCTCGGCGGTATGCTGATGGCGGGTATTGTGGTGGTGCTGATTGGTATGGTTGCGAATATCTTCCTGCAGCTGCCT   900
   T  T  R  K  D  M  S  F  L  G  G  M  L  M  A  G  I  V  V  V  L  I  G  M  V  A  N  I  F  L  Q  L  P   158

GCTCTGCATCTGGCGATCAGCGCGGTCTTCATTCTGATCTCCTCTGGCGCTATCTTGTTTGAAACCAGCAACATCATTCATGGCGGTGAGACGAACTATA  1000
A  L  H  L  A  I  S  A  V  F  I  L  I  S  S  G  A  I  L  F  E  T  S  N  I  I  H  G  G  E  T  N  Y  I   192

TTCGTGCCACGGTTAGCCTGTATGTTTCGCTGTACAACATCTTCGTCAGCCTGCTGAGCATTCTGGGCTTCGCTAGCCGCGATTAATCTCACCCGCTAAC  1100
   R  A  T  V  S  L  Y  V  S  L  Y  N  I  F  V  S  L  L  S  I  L  G  F  A  S  R  D  *                 219

ACTTTCATAGCCTCGCTTTATGCGGGGCTTTGTTTTTTGTTACACTGCCGCCAGTCTGATTAAGGTAGTAATAATTATGCTGATCTTCGAAGGTAAAGAG  1200
ATAGAAACGGATACCGAAGGCTATCTCAAAGAAAGCAGCCAGTGGAGTGGCCGCTGGCTGTGGTGATTGCAGAGAACGAAGGGATTTCGCTGTCACCGGA  1300
ACACTGGGAAGTGGTGCGTTTTGTGCGTGATTTCTATCTGGAATTC
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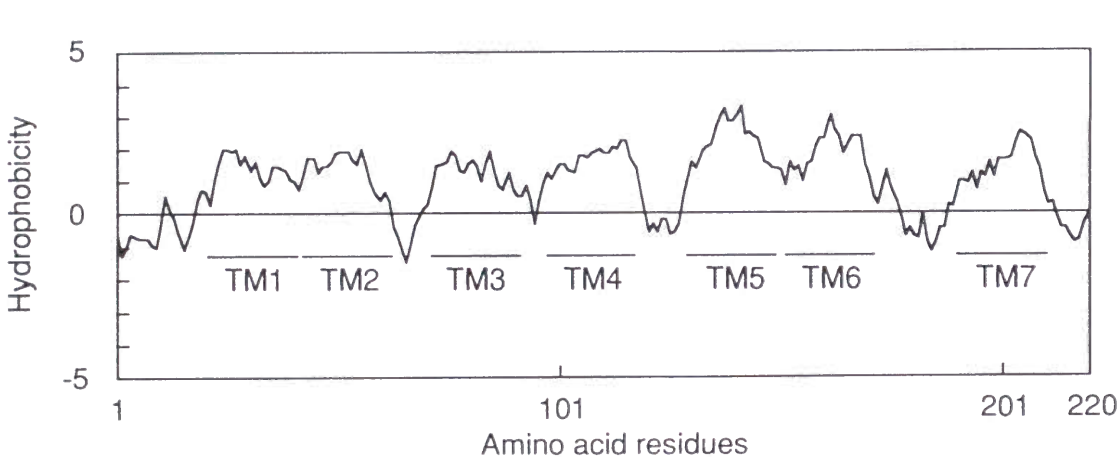
B



C

```
YccA            ·····MDRIVSSSHDRTSLLSTHKVLRN
YccA11          ·····MDRI·····SLLSTHKVLRN
N8-YccA11       MTMITPSLHDRI·····SLLSTHKVLRN
```

Fig. 29 Sequence and hydropathic profile of YccA
A. The nucleotide sequences of 1.3 kb *Eco*RI fragments from wild-type and *yccA11* mutant are shown. The residues deleted in the *yccA11* mutant are boxed. The amino acid sequences of YccA are similarly shown. The wild-type sequence agreed exactly with that determined by Tamura *et al.* (1984). Putative transmembrane segments are underlined. **B**. Hydropathy plots (Kyte and Doolittle, 1982) (window size of 10) of YccA. TM1-TM7 indicate transmembrane segments predicted by the TopPred program. **C**. The predicted amino acid sequences of the first cytoplasmic domain of YccA, YccA11, and N8-YccA11.

However, it did not show any detectable phenotypes.

Stability of excess SecY was examined by pulse-chase and immunoprecipitaion experiments. In SecY-overproducing "wild-type" cells, a fraction of SecY which is excess over SecE was degraded with a half-life of about 2 min (Fig. 30, open circles). In the *yccA11* mutant cells, the unstable fraction of SecY showed an increased half-life of about 7 min (closed squares). Note that an apparent increase in the initial labeling of SecY observed in the mutant cells was probably due to stabilization of nascent or newly-synthesized SecY molecules as already discussed.

Table II Effect of the *yccA11* mutation on accumulation of overproduced SecY-LacZα

Exp.	Host Strain*	Relevant genotype	Plasmid carried (in addition to pKY258†)	colony color‡
1	AD16	WT	–	–
2	AK897	WT	–	–
3	AK904	*yccA11*	–	++
4	AK990	*ΔhflK-hflC*	–	–
5	AK1396	*yccA11 ΔhflK-hflC*	–	–
6	AD16	WT	pKH135 (*yccA*)	–
7	AD16	WT	pKH189 (*yccA11*)	++
8	AK904	*yccA11*	pKH131 (divE, *yccA*)	+
9	AK904	*yccA11*	pKH135 (*yccA*)	+
10	AK904	*yccA11*	pKH198 (*ftsH*)	–
11	AD16	WT	pKH330 (*yccA-his$_6$-myc*)	–
12	AD16	WT	pKH331 (*yccA11-his$_6$-myc*)	++
13	AK990	*ΔhflK-hflC*	pKH330 (*yccA-his$_6$-myc*)	–
14	AK990	*ΔhflK-hflC*	pKH331 (*yccA11-his$_6$-myc*)	–

Cells were grown on L agar containing 40 μg/ml XG, 0.25 mM tPEG, 1 mM IPTG, and appropriate antibiotics at 37°C for 14 hours.

* All strains were derivative of AD16 (*Δpro-lac thi* /F' *lacI*^{q} Z^{M15} Y$^+$ *pro*$^+$).

† pKY258 was derived from pACYC184 and carried *secY-lacZα*.

‡ Colony color evaluated was shown by "–" representing pale blue, "++" representing dark blue, and "+" representing intermediate color. The degree of blue color indicates stability of the SecY-LacZα fusion protein (see also Kihara *et al.*, 1994 and Homma *et al.*, 1995).

The pKH135 (*yccA*$^+$) plasmid complemented the *yccA11* mutant phenotype only weakly (Table II, Exp. 9). On the other hand, introduction of plasmid (pKH189) carrying the *yccA11* mutant gene into wild-type cells resulted in significant stabilization of the SecY-LacZα fusion protein (Exp. 7). Thus, the *yccA11* mutation is partially dominant. Possibly, the mutant form of YccA interferes with the FtsH-dependent proteolysis of SecY by titrating out FtsH. In support of this notion, when FtsH was overproduced from plasmid (pKH198) in the *yccA11* mutant cells, the degradation of SecY-LacZα was restored to nearly the wild-type level (Exp. 10).

We found that the YccA11 effect on stability of SecY required the presence of HflKC. When Δ*hflK-hflC* mutation was introduced into the *yccA11* mutant, the half-life of SecY was shortened from about 7 min to about 2 min (Fig. 30, closed circles). Consistent with the pulse-chase result, SecY-LacZα did not overaccumulate in the *yccA11* Δ*hflK-hflC* double mutant cells (Table II, Exp. 5). These results indicate that YccA11 mutant protein interferes with the SecY-degrading function of FtsH, and this interference is somehow mediated by HflKC.

2. Physical interaction of the YccA11 protein with the FtsH-HflKC complex

Wild-type, Δ*hflK-hflC*, *yccA*::*kan*, and *yccA11* cells were labeled with [^{35}S]methionine and their membrane fractions were subjected to chemical crosslinking, using DSP. After crosslinking, proteins were solubilized in SDS, immunoprecipitated with anti-HflK antibodies, and separated by SDS-PAGE under reducing conditions in which linkages between the primary immunogen (HflK) and its crosslinked partners were cleaved. HflC and FtsH were the major crosslinked products (Fig. 31A, lane 1; see also Fig. 16). In addition, a number of faint protein bands (such as those marked by asterisks in Fig. 31A, lane 1) were observed. These proteins were specific in that they were not seen without DSP treatment (lane 2) or when Δ*hflK-hflC* cells were used (lane 3). Among them, a 23 kDa protein was specifically absent in the *yccA*::*kan*

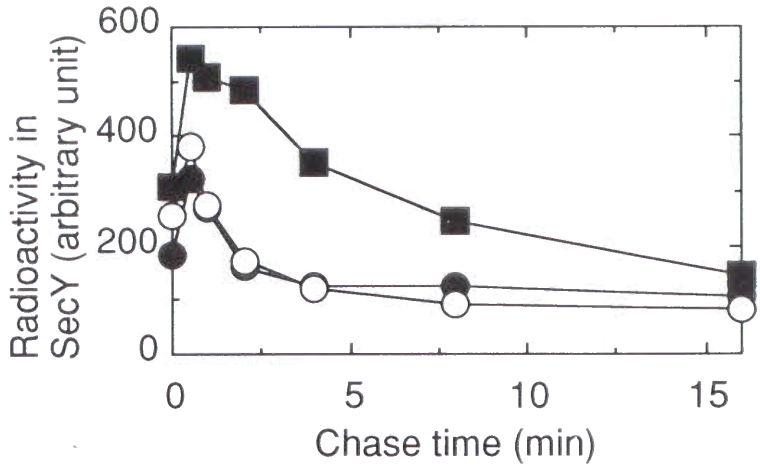


Fig. 30 Effects of the *yccA11* mutation on degradation of overproduced SecY. Cells of AK897 (wild-type)/pKY248 (p*lac-secY*) (open circles), AK904 (*yccA11*)/pKY248 (closed squares), and AK1396 (*yccA11* Δ*hflK-hflC*::*tet*)/pKY248 (closed circles) were grown at 37°C. Plasmid-encoded SecY was induced with IPTG and cyclic AMP for 10 min, and cells were pulse-labeled with [^{35}S]methionine for 0.5 min followed by chase for indicated periods. Radioactive SecY was immunoprecipitated, subjected to SDS-PAGE, and visualized. Radioactivities associated with SecY were quantitated.

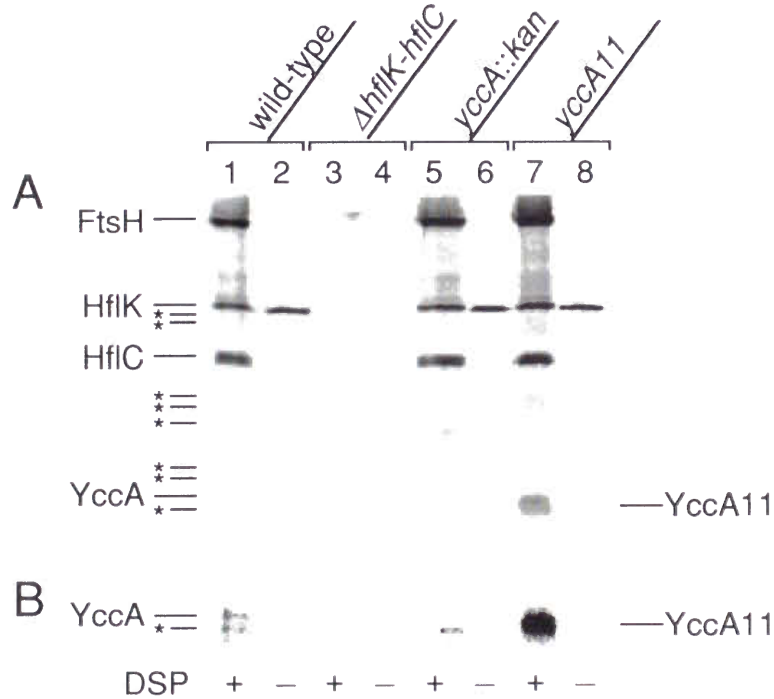


Fig. 31 Crosslinking of YccA with the FtsH/HflKC complex.
A. Cells of AD179 (wild-type; lanes 1 and 2), AK1441 (Δ*hflK-hflC*; lanes 3 and 4), AK1485 (*yccA*::*kan*; lanes 5 and 6) and AK1405 (*yccA11*; lanes 7 and 8) were labeled at 37°C with [^{35}S]methionine for 30 min. Total membrane fractions were treated with DSP (lanes 1, 3, 5 and 7) or its solvent, DMSO (lanes 2, 4, 6 and 8). Membrane proteins were solubilized with SDS, and subjected to immunoprecipitation using anti-HflK. Immunoprecipitates were solubilized in SDS sample buffer containing 2-mercaptoethanol, separated by SDS-PAGE, and visualized using a Bioimaging Analyzer BAS2000 (Fuji Film). FtsH, HlfK, HflC, YccA and YccA11 are indicated, while asterisks indicate specific but minor crosslinked polypeptides. **B**. The YccA region of the gel was exposed longer and shown separately for clarity.

mutant cells (Fig. 31A and B, lane 5). When the *yccA11* mutant cells were used, a protein of slightly faster migration was found to be strongly crosslinked with HflK (Fig. 31A and B, lane 7). Taking the fact that the YccA11 mutant protein, which is missing 8 amino acids, was stable while the wild-type YccA is unstable (see below), these results strongly suggest that the 23 kDa protein and the slightly smaller counterpart observed in the mutant cells represented the wild-type and the mutant form of the YccA proteins, respectively.

To distinguish between mere proximity and specific binding, co-immunoprecipitation of the YccA protein with HflKC was examined (Fig. 32). The labeled membranes were solubilized with digitonin and subjected to immunoprecipitation with anti-HflKC under non-denaturing conditions. FtsH was efficiently co-immunoprecipitated under these conditions (lane 1). Again, a number of weak bands were observed, many of which disappeared when the antiserum was preincubated with an excess of HflKC protein (lane 2) or when the membrane extract from Δ*hflK-hflC* cells was used (lane 3). Whereas these proteins can be regarded specific (asterisks), those observed even under the latter conditions (lanes 2 and 3) were non-specific backgrounds (dots). The experiment using the *yccA11* mutant yielded a prominent co-immunoprecipitation product (lane 4) that was identical in the electrophoretic mobility with the YccA11 mutant protein observed in the crosslinking experiments. A band corresponding to the wild-type YccA protein was also observed, albeit it was weak (lane 1).

3. YccA interacts both with FtsH and HflKC

We also carried out crosslinking experiments using anti-FtsH to bring down the products (Fig. 33). In wild-type cells, anti-FtsH brought down two major crosslinked products, HflK and HflC (lane 1). A faint crosslinked band of YccA was observed again. Crosslinking between FtsH and YccA persisted (although weakly) in the absence of HflKC (lane 5). The crosslinking of the YccA11 mutant protein was

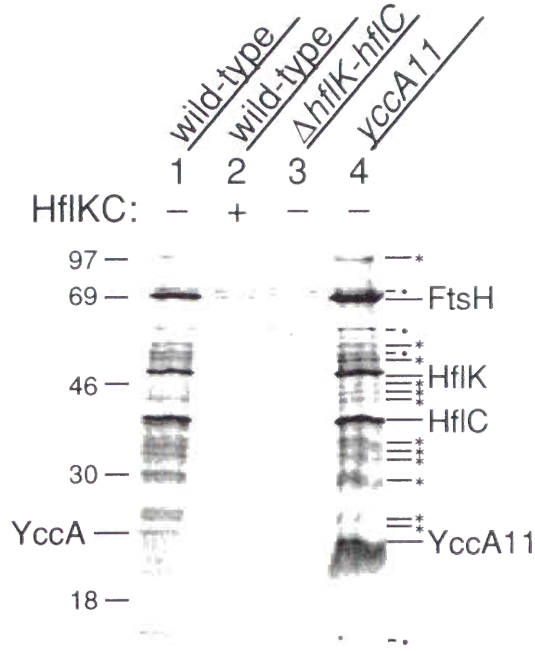


Fig. 32 Co-immunoprecipitaion of YccA with FtsH/HflKC.
Cells of AD179 (wild-type; lanes 1 and 2), AK1441 (*ΔhflK-hflC*; lane 3), and AK1405 (*yccA11*; lane 4) were labeled with [^{35}S]methionine. Membrane proteins were solubilized under non-denaturing conditions and precipitated with anti-HflKC in the absence (lanes 1, 3, and 4) or in the presence (lane 2) of excess purified HflKC. Immunoprecipitates were separated by SDS-PAGE and visualized by autoradiography using a Fujix bioimaging analyzer BAS2000. Asterisks were anti-HflKC specific but unidentified proteins, and dots were non-specific background.

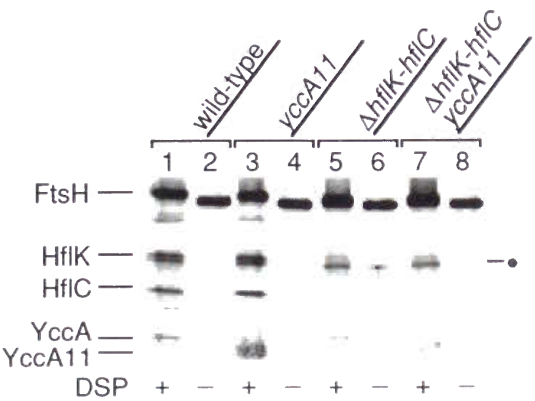


Fig. 33 Crosslinking between YccA and FtsH.
Cells of AD179 (wild-type; lanes 1 and 2), AK1405 (*yccA11*; lanes 3 and 4), AK1441 (*ΔhflK-hflC*; lanes 5 and 6) and AK1748 (*ΔhflK-hflC yccA11*; lanes 7 and 8) were labeled at 37°C with [^{35}S]methionine. Total membrane fractions were treated with DSP (lanes 1, 3, 5 and 7) or DMSO (lanes 2, 4, 6 and 8). Membrane proteins were solubilized with SDS and subjected to immunoprecipitation using anti-FtsH. Immunoprecipitates were solubilized in SDS sample buffer containing 2-mercaptoethanol, separated by SDS-PAGE, and visualized using a Bioimaging Analyzer BAS2000 (Fuji Film). The proteins shown by dot was probably a nonspecific background.

greatly reduced in the absence of HflKC, although it was not completely abolished (compare lane 7 with lane 3). The fact that crosslinking between FtsH and YccA (or YccA11) persisted in the absence of HflKC suggest that there is a direct interaction between YccA and FtsH. On the other hand, the observation that crosslinking between YccA11 and FtsH was markedly reduced in the absence of HflKC is in apparent accordance with the fact that the *in vivo* ability of the YccA11 protein to interfere with proteolysis of SecY was abolished when *hflK* and *hflC* had been deleted. These results are consistent with the notion that the YccA11 mutant protein inhibits FtsH through direct interaction with both HflKC and FtsH.

We studied whether HflKC can associate directly with YccA, using purified proteins *in vitro*. We constructed a YccA-His_6-Myc fusion protein, and purified it from the NP40-solubilized membrane fraction prepared from YccA-His_6-Myc overproducing cells, using Ni-NTA-agarose affinity chromatography (Fig. 34A). A purified preparation of HflKC did not bind to Ni-NTA agarose as they were found in the flow-though fraction with some tailing into the first wash fraction (Fig. 34B, lanes 2 and 3). In contrast, when the same preparation of HflKC was pre-mixed with YccA-His_6-Myc in digitonin-containing buffer, not only YccA-His_6-Myc but a significant fraction of HflK and HflC were retained in the column and eluted with 250 mM imidazole (lane 12). Thus, HflKC was able to associate with YccA-His_6-Myc. These results, taken together, suggest that both FtsH and HflKC can recognize and bind to YccA, a membrane-embedded substrate of FtsH (see below).

4. YccA is a substrate of FtsH

The apparent yield of crosslinking between YccA and FtsH/HflKC complex was much lower than that between YccA11 and FtsH/HflKC. This raised a possibility that wild-type YccA was a substrate of FtsH and unstable, while YccA11 was not degraded by FtsH. We examined the stability of YccA-His_6-Myc and its mutant version, YccA11-His_6-Myc. When YccA11-His_6-Myc was overproduced in wild-type

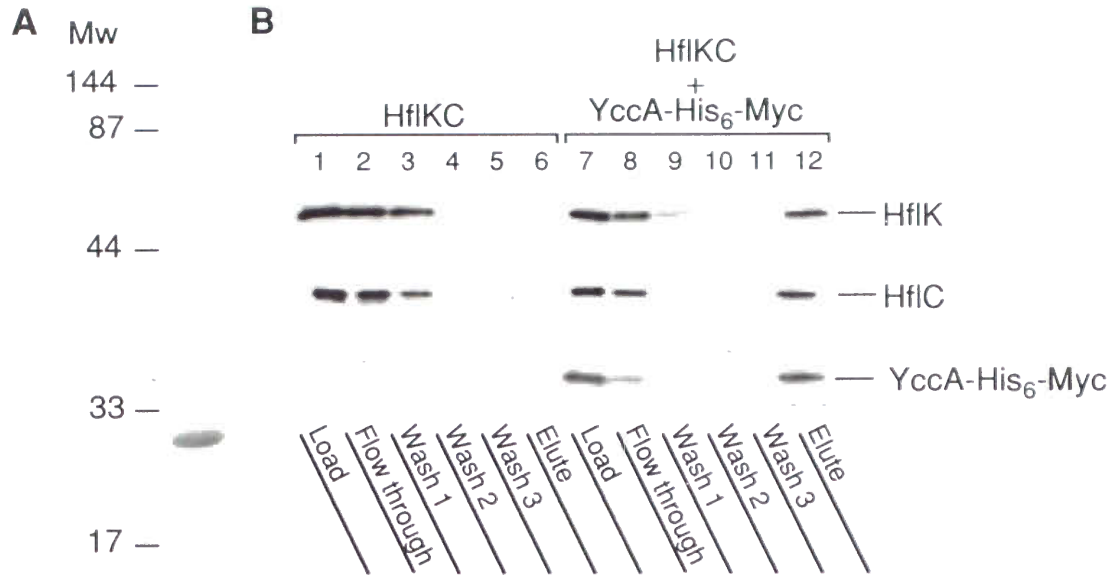


Fig. 34 Purified HflKC and YccA can associate *in vitro*.
A. YccA-His_6-Myc was purified as described in Materials and Methods, and its coomassie brilliant blue-stained SDS-PAGE pattern is shown. B. YccA-His_6-Myc (1.3 μg) alone (lanes 1-6) or a mixture of YccA-His_6-Myc (1.3 μg) and HflKC (1.3 μg) (lanes 7-12) was incubated at 0°C for 1 hr and applied to a Ni-NTA-agarose column (load, lanes 1 and 7; flow-through, lanes 2 and 8). The column was washed three times (lanes 3-5 and 9-11) and eluted with 250 mM imidazole (lanes 6 and 12). Proteins in each fraction were separated by SDS-PAGE and visualized by immunoblotting using antisera against HflKC (upper and middle panels) or anti-Myc antibodies (lower panel).

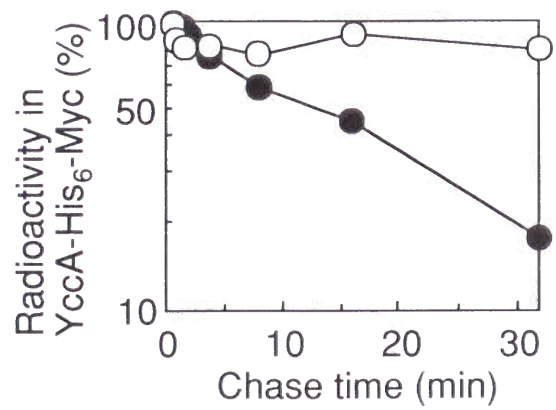


Fig. 35 YccA-His_6-Myc is a substrate of FtsH *in vivo*.
Cells of AR796 (*ftsH*$^+$; closed circles) and AR797 (*ftsH1*; open circles), each bearing pKH330 (p*yccA-his*$_6$*-myc*), were grown at 30°C and shifted to 42°C for 1 hr. Cells were pulse-labeled with [^{35}S]methionine for 1 min followed by chase for indicated time periods. YccA-His_6-Myc was immunoprecipitated, separated by SDS-PAGE, and visualized. Radioactivities associate with YccA-His_6-Myc were quantitated. Values relative to that at 0.5 min chase point are shown for each curve.

cells, the dominant SecY-stabilization effect was observed. But such an effect was not observed in Δ*hflK-hflC* cells (Table II, Exp. 12 and 14). Thus, attachment of the tags did not affect the SecY-stabilization ability of YccA11.

Pulse-chase and immunoprecipitation experiments using anti-Myc antibodies showed that YccA-His_6-Myc was unstable with a half-life of about 15 min in wild-type cells (Fig. 35, closed circles), whereas it was stable in the *ftsH1* temperature-sensitive mutant (open circles) at 42°C. YccA-His_6-Myc was degraded also in Δ*hflK-hflC* cells (Fig. 36, closed squares) as in the *hfl*$^+$ cells (closed circles). However, the mutant form, YccA11-His_6-Myc, was not appreciably degraded whether it was expressed in wild-type cells or in the Δ*hflK-hflC* cells (open circles and open squares).

These results indicate that YccA is a substrate of the FtsH proteolytic system. The YccA-FtsH/HflKC complex we observed *in vivo* should have represented their transient interaction in the course of proteolysis. The lack of degradation may explain the apparently stronger interaction observed between YccA11 and FtsH/HflKC. We assume it likely that the internal deletion in YccA11 abolished a site required for proteolysis by FtsH, while the mutation did not impair binding between YccA11 and FtsH/HflKC. It should be noted, however, that the weaker crosslinking between the stable YccA11 protein and FtsH in the absence of HflKC (Fig. 33, lane 7) was not ascribable to a reduction in the amount of the former. Thus, efficient binding between FtsH and YccA requires the mediation by HflKC.

The *yccA11* deletion of 8 amino acids makes the YccA11 protein to be refractory to the degradation by FtsH. It seemed that the 8 amino acid residues were important for the degradation intiation. All the cytoplasmic regions of YccA are small in their sizes, with the amino-terminal one the largest. We thought it possible that the size of the amino-terminal region may be critical for YccA to be a substrate of FtsH. We constructed a fusion protein, N8-YccA11-His_6-Myc, in which an unrelated sequence of 8 amino acids were attached to the N-terminus of YccA11-His_6-Myc (Fig. 29C). It was found that N8-YccA11-His_6-Myc was as unstable as YccA in wild-type cells (Fig.

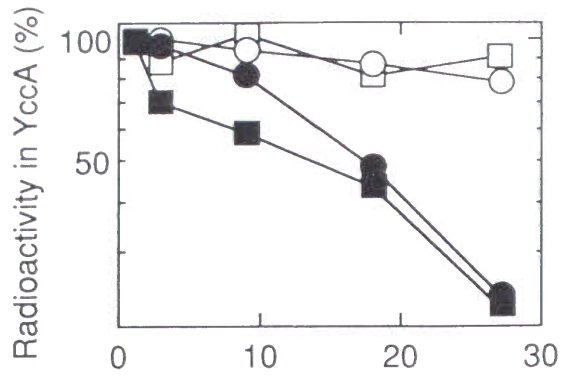


Fig. 36 Stability of YccA-His_6-Myc and YccA11-His_6-Myc in wild-type and in *ΔhflK-hflC* mutant cells.
Cells of AD16 (wild-type)/pKH330 (*yccA-his$_6$-myc*) (closed circles), AK990 (*ΔhflK-hflC*)/pKH330 (closed squares), AD16/pKH331 (*yccA11-his$_6$-myc*) (open circles), and AK990/pKH331 (open squares) were pulse-labeled with [^{35}S]methionine for 1 min and chased with unlabeled methionine for 1, 3, 9, 18, and 27 min. Proteins were immunoprecipitated with anti-Myc antibodies and separated by SDS-PAGE. Radioactivities associated with YccA-His_6-Myc and YccA11-His_6-Myc were quantitated. Values relative to that at 1 min chase point are shown for each curve.

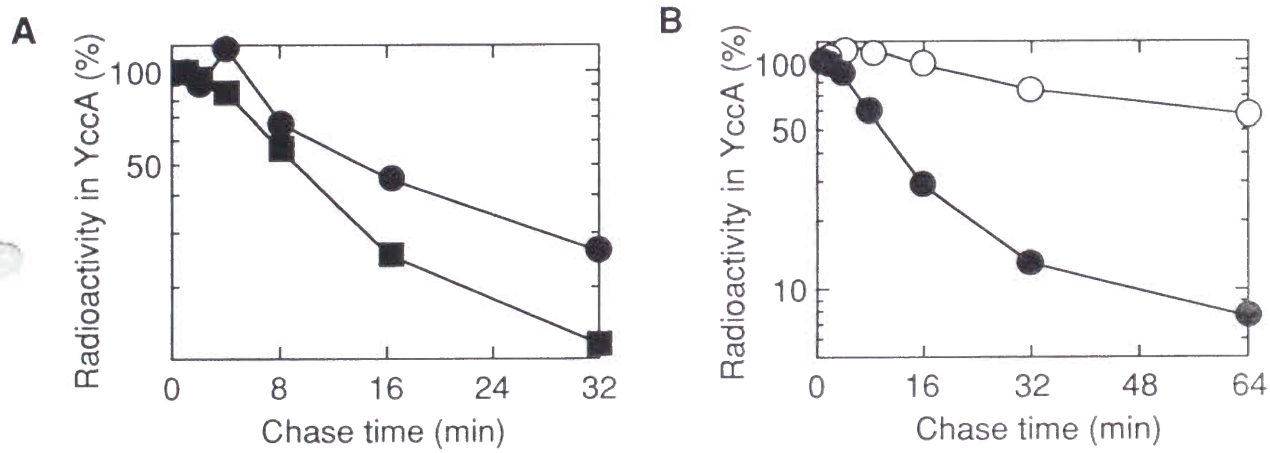


Fig. 37 Stability of N8-YccA11-His_6-Myc.
A. Cells of AD16 (wild-type)/pKH356 (*N8-yccA11-his$_6$-myc*) (closed circles) and AK990 (*ΔhflK-hflC*)/pKH356 (closed squares) were grown at 37°C, induced with 1mM IPTG and 3 mM cAMP for 10 min, pulse-labeled with [^{35}S]methionine for 1 min, and chased with unlabeled methionine for 1, 2, 4, 8, 16, and 32 min. **B**. Cells of AR796 (wild-type)/pKH356 (closed circles) and AR797 (*ftsH1*)/pKH356 (open circles) were grown at 30°C and shifted to 42°C. Cells were induced with 1 mM cAMP for 10 min, pulse-labeled for 1min, and chased for 1, 2, 4, 8, 16, 32, and 64 min. Proteins were immunoprecipitated with anti-Myc antibodies and separated by SDS-PAGE. Radioactivities associated with N8-YccA11-His_6-Myc were quantitated. Values relative to that at 1 min chase point are shown for each curve.

37A, closed circles). The N8-YccA11-His_6-Myc was degraded slightly faster in *ΔhflK-hflC* (closed squares) than in *hfl*$^+$ cells. It was stabilized in the *ftsH1* mutant cells (Fig. 37B, open circles). Thus, the sequence deleted by *yccA11* may not itself be essential for the proteolysis, provided that the cytoplasmic tail at the amino terminus is long enough.

5. Differential effects of the *yccA11* mutation on stability of different FtsH substrates

We addressed the generality of the proteolysis interference caused by the YccA11 mutant proteins. Recently, Akiyama *et al.* (1996b) showed that subunit *a* of proton ATPase F_0 sector is a substrate of FtsH when it fails to make a complex. The subunit *a* is hydrophobic and embedded into the membrane by 8 transmembrane segments (Lewis *et al.*, 1990). Pulse-chase and immunoprecipitaion experiments showed that overproduced F_0 subunit *a* was stabilized in *yccA11* cells (Fig. 38A, closed squares), whereas it was degraded with a half-life of about 1-2 min in wild-type cells (closed circles). The F_0 *a* subunit was also stabilized by the *hflK13* (open diamonds) and the *hflC9* (open triangles) SecY-stabilzing mutations. In contrast, λ CII protein, overproduced from a plasmid (pKH274), was degraded with a half-life of about 10 min both in the wild-type cells and in the *yccA11* cells (Fig. 38B, closed circles and closed squares). The *yccA11* mutant did not show any high frequency lysogenization (Hfl) phenotype; the frequency of lysogenization of λ was 1.2% in *yccA11* cells, a value similar to that in the wild-type cells (1.6%). Thus, the *yccA11* mutation did not affect stability of the CII protein under physiological conditions. The *hflK13* and *hflC9* mutations did not affect CII stability either as shown in Table I. These results indicate that proteolysis catalyzed by FtsH can be differentially affected by a mutant form of YccA, as well as by the gain of function mutations in *hflK* or *hflC*. YccA, SecY and F_0 subunit *a* appear to share a common recognition pathway by the FtsH proteolytic system, but the λ CII protein may enter the system via a different route.

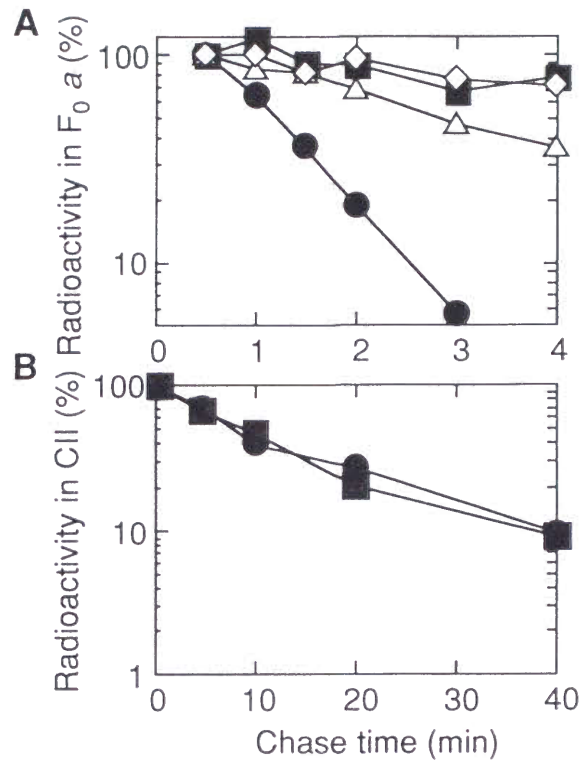


Fig. 38 Effects of the *yccA11* mutation on stability of F_0 *a* and λ CII.

A. Cells of AK1653 (*yccA*$^+$; closed circles), AK1654 (*hflC9*; open triangles), AK1655 (*hflK13*; open diamonds), and AK1656 (*yccA11*; closed squares), each bearing pSTD181 (*atpB* for subunit *a* of the H$^+$-ATPase F_0 sector), were grown at 37°C and induced with 1 mM IPTG and 5 mM cAMP for 10 min. Cells were pulse-labeled for 0.5 min and chased for 0.5, 1, 1.5, 2, 3, and 4 min. Radioactive subunit *a* was immunoprecipitated, separated by SDS-PAGE, and quantitated. Values relative to that at 0.5 min chase point are shown for each curve. **B**. Cells of AK897 (*yccA*$^+$; closed circles) and AK904 (*yccA11*; closed squares), each bearing pKH274 (*para-cII*), were grown at 37°C and induced with 0.4% arabinose for 10 min. Cells were pulse-labeled for 0.5 min and chased for 0.5, 5, 10, 20, and 40 min. Total proteins were separated by SDS-PAGE, and radioactivities associated with CII were quantitated. Values relative to that at 0.5 min chase point are shown for each curve.

§ 3 Discussion

1. FtsH is the protease responsible for the degradation of SecY and λ CII

In this study, we found that mutations in *ftsH* stabilized oversynthesized SecY subunit of protein translocase. This stabilization is due to a loss of FtsH function, and overproduction of the wild-type FtsH protein accelerated the degradation. The temperature-sensitive *secY24* mutant produces SecY that is only weakly associated with SecE and subject to degradation at 42°C (Baba *et al.*, 1994). The *ftsH* mutations suppressed the temperature sensitivity by stabilizing the altered form of SecY. We were able to isolate a number of mutants with decreased *ftsH* expression or with an altered form of FtsH using selection/screening based on suppression of *secY24* and stabilization of oversynthesized SecY (Fig. 9). These results indicate that FtsH is required for degradation of uncomplexed forms of SecY. Direct evidence that FtsH is the SecY-degrading protease has been provided using purified FtsH and SecY proteins; FtsH directly degrades SecY in an ATP-dependent manner *in vitro* (Akiyama *et al.*, 1996a; see also Fig. 22). Tomoyasu *et al.* (1995) also showed that purified FtsH degrade σ^{32}, a heat shock σ subunit of RNA polymerase. They proposed that FtsH is a zinc-metalloprotease, since Zn^{2+} stimulated the σ^{32}-degrading activity of FtsH whereas heavy metal chelating agent, o-phenanthroline, inhibited the activity. Indeed, FtsH possesses a zinc-binding motif. We demonstrated in this study that purified FtsH catalyzes the degradation of λ CII protein (Fig. 23). The CII-degrading activity of FtsH, like its σ^{32}-degrading activity, was inhibited by EDTA but not by PMSF, a serine protease inhibitor (data not shown).

2. HflKC forms a complex with FtsH and regulate its proteolytic activity

Our screening for mutants in which overproduced SecY was stabilized yielded predominantly recessive mutations affecting *ftsH* (Fig. 9). In addition, we isolated partial dominant mutations in the *hflK* and *hflC* genes (Fig. 13). The genes thus

identified coincided with those previously known as *hfl*. *E. coli hfl* mutations are known to increase lysogenization frequency of λ by stabilizing the CII protein (Hoyt *et al.*, 1982; Banuett *et al.*, 1986). However, the Δ*hflK-hflC* mutation did not stabilize excess SecY. Thus, HflKC is not positively required for the degradation of SecY. The mutational effects of *hflK13* and *hflC9* were suppressed by overproduction of FtsH, suggesting that these mutant forms of HflK or HflC antagonize the FtsH activity. These results raised the possibility that even the wild-type HflKC protein interacts with FtsH and inhibits its proteolytic activity. Crosslinking, co-immunoprecipitation, histidine tagging and gel filtration experiments showed that FtsH and HflKC indeed form a complex *in vivo* and *in vitro*. Judging from the *in vitro* effects of HflKC on FtsH-mediated degradation of SecY as well as *in vivo* effects of an *hflK-hflC* deletion on the degradation of SecY24 protein, we propose that HflKC negatively regulates FtsH, and that the HflK13 and the HflC9 mutant proteins have gained stronger inhibitory activities. That the overproduction of wild-type HflK or HflC subunit suppressed or "complemented" the respective *hflK13* or *hflC9* mutation may be explained as follows. In the formation of the HflK-HflC complex, the overproduced wild-type subunit competes with the mutated subunit, leading to a lowered concentration of the complex that contains a mutated subunit. Since such decrease will be in proportion to the extent of overproduction of the wild-type subunit, the strongly inhibiting complex will be sufficiently diluted by the moderately inhibiting wild-type complex.

3. HflKC is not a CII-degrading protease

Contrary to the previous report that HflKC is a protease (Cheng *et al.*, 1988), the purified HflKC preparation did not show any CII-degrading activities in our experiments. Instead, it was ihibitory against the FtsH-dependent proteolysis *in vitro*. These results raised a serious question about the validity of the proposal that HflKC is a CII-degrading protease (Hoyt *et al.*, 1982; Banuett *et al.*, 1986; Cheng *et al.*,

1988). This proposal has been supported by the following observations. First, Hoyt *et al.* (1982) and Banuett *et al.* (1986) showed that CII protein was stabilized in *hflA* mutants *in vivo*. Second, as mentioned above, Cheng *et al.* (1988) purified HflKC and demonstrated its serine-protease like activity to degrade CII. Third, HflC contains a sequence motif similar to that found in the active site region of the ClpP protease (Noble *et al.*, 1993), an ATP-dependent serine protease subunit in *E. coli*.

We showed that the CII protein synthesized from the cloned gene in the absence of other λ gene products (or the λ genome) was not stabilized by the *hflK-hflC* mutations except under certain temperature conditions. The reason why the temperature conditions affect the results is unknown, but it should be noted that transcription of *ftsH* is partially subject to heat shock control by σ^{32} (Herman *et al.*, 1995). In addition, different temperature conditions affect cellular compositions of partially denatured proteins and molecular chaperones, thus potentially affecting proteolytic systems and their substrates. On the other hand, we could reproduce the previous report (Hoyt *et al.*, 1982) that CII protein is stabilized in Δ*hflK-hflC* cells when it was synthesized from the λ genome under the conditions of infection. The difference from the situation of cloned CII could be explained by the different expression levels, as well as by the presence or absence of other λ gene products and the λ DNA to which CII may bind. In particular, the λ *cIII* gene product is an inhibitor of FtsH (Hoyt *et al.*, 1982; Herman *et al.*, 1997) and should affect the CII stability.

We found that overproduction of HflKC in wild-type cells did not accelerate the degradation of CII, but rather resulted in its stabilization (Fig. 25). This result does not agree with the previous report that HflKC can degrade CII without a supply of other protein factors. In contrast, a loss of *ftsH* function stabilized CII almost completely, whereas its overexpression accelerated the CII degradation *in vivo*.

We failed to detect any CII-degrading activity in our preparation of HflKC. The exact reason for the discrepancy with the results of Cheng *et al.* (1988) is unknown,

but it is generally difficult to exclude the possibility of contaminations. For instance, we detected a serine protease-like CII-degrading activity in crude membrane fractions prepared from the Δ*hflK-hflC* cells. It seems possible that the CII degradation activity described by Cheng *et al.* (1988) was actually due to such an unidentified protease that contaminated the HflKC preparation.

Although Noble *et al.* (1993) claimed that HflC contains a domain resembling the catalytic domain of ClpP protease, the putative active site serine is not conserved among its homologs. Moreover, *H. influenzae* HflC lacks a 36 amino acids which overlaps the ClpP-like domain. We demonstrated that HflCΔ165-200, which has a deletion of the proposed protease active site, can function as a λ lysogenization controller.

All these results are inconsistent with the hypothesis that HflKC is a protease responsible for the degradation of CII. In addition, although it has been believed that the C-terminal large domains of HflK and HflC are cytoplasmically disposed (Gottesman, 1996), we found that they essentially reside on the periplasmic side of the plasma membrane. While it is clear that FtsH is the protease that degrades CII, these results indicate that HflKC is not a CII-degrading protease. Its association with FtsH should have some regulatory significance.

4. YccA is a natural substrate of FtsH

We have identified a SecY-stabilizing mutation in *yccA*, an open reading frame of previously unknown function, and identified the product of this gene. The *yccA* gene product has seven hydrophobic segments, which most probably traverse the membrane. It is one of the most hydrophobic class of proteins (114 hydrophobic residues (Ala, Ile, Leu, Val, Met, Trp, and Phe) in a total of 219 residues) in *E. coli*. The positive inside rule (von Heijne, 1986) predicts that its amino terminal region faces the cytoplasm, while its carboxy terminal region faces the periplasm.

The *yccA11* mutation, causing a deletion of 8 amino acids in the amino terminal

hydrophilic region of YccA, is similar to the *hflK13* and the *hflC9* mutations in that it is partially dominant with respect to the phenotype of stabilization of excess SecY protein. Thus, these mutant proteins somehow interferes with the FtsH-dependent proteolysis of SecY. However, the mechanisms underlying the interference appear to be different for the *hflKC* and the *yccA* mutations. Although the deletion of *hflK-hflC* accelerated the degradation of a mutant form of SecY, the chromosomal deletion of *yccA* did not (data not shown). The HflKC complex is stable in the cell. While free HflK and HflC subunits are unstable, their degradation is not FtsH-dependent (data not shown). Thus, HflKC is not itself a substrate of FtsH. In contrast, the histidine-tagged YccA protein was degraded by the FtsH proteolytic system, whereas its YccA11 mutant version was stable. The chromosomally encoded YccA protein was crosslinked with FtsH/HflKC, and such crosslinking product was increased strikingly by the *yccA11* mutation. We interpret all of these experimental results to mean that YccA is a natural substrate of FtsH. Since YccA is degraded eventually, its association with the FtsH/HflKC complex is transient. In contrast, the YccA11 mutant protein is stably associated with FtsH/HflKC. Thus, the site for the recognition by FtsH/HflKC and that for the initiation of proteolysis are differentially located in the YccA sequence and the *yccA11* deletion only abolishes the latter. The mutant protein will then compete with other substrates, such as SecY, of FtsH.

We observed a weak "complementation" effect of the overproduced YccA protein on the degradation of overproduced SecY (Table II, Exp. 9). This might be simply due to a dilution effect by the normally degraded wild-type protein. If the wild-type YccA protein is indeed a natural substrate of FtsH, its instability might have some physiological significance, but the absence of the disruption phenotypes (data not shown) precludes an easy assignment of the physiological roles played by YccA. We observed several other proteins that were crosslinked with FtsH/HflKC; they might well be additional substrates of this proteolytic system.

5. The mechanism of degradation of membrane proteins

Accumulation of abnormal proteins in the membrane may lead to uncontrolled collapse of chemical gradients. Thus, "quality control" of the membrane will be important. In fact, we showed that accumulation of uncomplexed SecY caused growth disadvantage and suboptimal protein export efficiency. Our knowlegde about degradation of membrane proteins is very limited, and in this respect FtsH, which catalyzes proteolysis of integral membrane proteins at least of SecY, F_0 *a*, and YccA, provides a unique opportunity to investigate into the mechanism and regulation of membrane protein degradation.

Evidence suggests that ATP-dependent proteases degrade cytoplasmic proteins at multiple sites, probably in processive manners, without release of high molecular weight intermediates (Thompson *et al.*, 1994). *In vivo*, released short polypeptides might further be degraded into free amino acids by peptidases and/or energy-independent proteases (Gottesman, 1996). ATP-dependent processive degradation includes ClpAP-mediated degradation of α-casein (Thompson *et al.*, 1994), Lon-mediated degradation of λ N (Maurizi, 1987) and F plasmid CcdA proteins (van Melderen *et al.*, 1996). We also observed that FtsH degraded [^{35}S]methionine labeled CII to produce low molecular weight (3-5 kDa) degradation products which appeared as smear bands in SDS-PAGE (data not shown). Bukau and his co-workers found that FtsH cleaves σ^{32} processively and produces small peptides with an average size of around 2 kDa (personal communication).

FtsH can degrade both soluble proteins like CII and σ^{32} and membrane proteins such as SecY, F_0 *a* and YccA. In the course of degradation of SecY, FtsH did not accumulate any detectable intermediates either *in vivo* or *in vitro*. Since FtsH is only anchored to the membrane via its N-terminal region and its ATPase/protease domain is located essentially in the cytosol, FtsH will meet difficulties when it attempts to degrade a transmembrane or periplasmic region of an integral membrane protein in

processive manners. In this case, the only solution might be either that the cytosolic domain of FtsH migrates into the membrane or that FtsH pulls out the target portion out of the membrane. Since the former possibility seems more unlikely than the latter possibility, we must consider the possibilty of dislocation of a membrane-embedded substrate out of the membrane. These considerations lead to a tempting possibility that the FtsH induces "retrograde" dislocation of the transmembrane and periplasmic domains of membrane proteins for their degradation in the cytosol, where the protease active site of FtsH is located. Recently, it has been shown that abnormal proteins can be dislocated out of the endoplasmic reticulum for degradation by the ubiquitin-proteasome system in the cytosol of eukaryotic cells (Hiller *et al.*, 1996; Wiertz *et al.*, 1996; Biederer *et al.*, 1997). Of course, another possibility is not excluded that FtsH cleaves a membrane protein only at its cytosolic domains and this is followed by release of the transmembrane and periplasmic portions for further degradation by other peptidases and proteases residing either in the periplasm or in the cytoplasm.

Although we do not have any direct evidence supporting the dislocation model, it at least merits further discussions. The 8 amino acids deleted by the *yccA11* mutation seem to be necessary for the initiation of degradation. YccA contains only very small cytoplasmic regions; 23, 6, 5 and 18 amino acid residues are assigned by the TopPred program for cytoplasmic regions 1-4 (C1-C4), respectively. In addition, the cytoplasmic regions other than C1 are tethered at both ends to the membrane. Therefore, only the C1 region may be of sufficient length and flexibility such that it is recognized by the cytosolic ATPase or protease domain of FtsH. Attachment of an unrelated sequence of 8 amino acids to the amino-terminus allowed the protein again to be degraded by FtsH. Thus, the size of the cytoplasmic domain seems to be crucial for the YccA protein for the degradation initiation by FtsH. Since we could not detect any degradation intermediates with a molecular weight predicted for the C1-cleaved YccA, it is likely that once degradation starts from the C1 domain, it

proceeds to degrade other domains of YccA. It has been shown that FtsH has an ability to bind to a denatured protein, such as alkaline phosphate. Degradation of a membrane protein, such as YccA, could be initiated by binding of the cytoplasmic domain of FtsH to a cytosolic and unstructured segment of the substrate. This cytosolic protein interaction might then activate the hypothetical dislocation activity of FtsH and subsequent presentation of the other portions of the substrate to the protease active site of FtsH.

6. The role of ATP-hydrolysis in protein degradation

The energy of ATP hydrolysis is not required for hydrolysis of a peptide bond, and the roles of ATP binding and hydrolysis in promoting proteolysis by ATP-dependent proteases are only partially understood. Several different roles played by ATP hydrolysis have been reported or discussed (for reviews, Goldberg, 1992; Gottesman and Maurizi, 1992). For instance, ATP might control an ATP-dependent protease by changing its conformation in terms of substrate accessibility to the protease active sites. ATP binding produces an active conformation of the Lon protease (Goldberg and Waxman, 1985; Waxman and Goldberg, 1986). ATP binding to ClpA and HslU promotes their complex formation with their respective protease subunit, ClpP and HslV (Maurizi, 1991; Huang and Goldberg, 1997). ATP hydrolysis could also provide the energy to disrupt secondary or tertiary stuructures of substrate proteins rendering them suspectible to degradation. Interestingly, although degradation of CcdA by Lon requires ATP hydrolysis, CcdA41, a carboxyl-terminal fragment of 41 amino acids which lacks the α-helical structure normally present in the wild-type CcdA, can be degraded without ATP hydrolysis (van Melderen *et al.*, 1996). The energy of ATP hydrolysis could be used for translocation of the cleavage sites on a substrate into the active site to ensure processive degradation. Gottesman and Maurizi (1992) proposed that the energy of ATP hydrolysis is used to screen for appropriate substrates. Such "energy-dependent scanning" has been proposed in

other systems. For example, UvrA binds to DNA and moves along it until damaged DNA sites are encountered (Thiagalingam and Grossman, 1991).

FtsH might use ATP and its hydrolysis in some or all of these contexts. Matsuzawa and co-workers found that purified cytoplasmic domain of FtsH can degrade σ^{32} independently of ATP *in vitro* (personal communication). Since N-terminal transmembrane region mediates the interaction between the FtsH subunits (Akiyama *et al.*, 1995), and the water-soluble form of FtsH is monomeric (H. Matsuzawa, personal communication), it is possible that ATP change the oligomeric state of FtsH to regulate its activity. We observed an ATP-dependent conformational change in FtsH as well as a polypeptide-binding ability of this protein (Akiyama *et al.*, submitted). In addition, ATP seems to induce the complex formation between FtsH and HflKC (Fig. 19), suggesting a possibility that ATP is involved in the HflKC-mediated regulation of FtsH. Finally, our speculation about the retrograde movement of a membrane-integrated portion of a membrane protein substrate points to the ATP utilization in this interesting but hypothetical process.

7. Role of HflKC in regulating FtsH

In vitro results indicated that HflKC negatively regulates the FtsH activities. It seems that HflKC differentially inhibits the protease activities against different substrates. There might be two classes of FtsH substrates: first class includes SecY and F_0 *a*, both of which are membrane proteins, and the second class includes CII in the cytoplasm. Although SecY and F_0 *a* are stabilized in the *hflK13* and the *hflC9* mutant cells (Fig. 14 and Fig. 38), degradation of the λ CII protein, a cytosolic substrate of FtsH, is unaffected (Table I). The *yccA11* mutation exerted very similar effects on stabilities of different proteins (Fig. 38). In contrast, the absence of HflKC or a deletion of a periplasmic segment of FtsH stabilized CII but not SecY (Fig. 25; Y. Akiyama, unpublished results). Both HflK and HflC have large periplasmic domains and their cytoplasmic portions are only 3 amino acid-long (HflC) or 79 amino acid-long

(HflK) (Fig. 27). Thus, functional interaction between FtsH and HflKC, which requires the periplasmic region of FtsH, may negatively modulate the FtsH's activity. We propose that HflKC preferentially down regulates the FtsH's proteolytic activity against the SecY-class of proteins. In the absence of HflKC, FtsH is now directed toward the SecY-like substrates, and, as a consequence, CII-like substrates may have decreased opportunities of degradation by FtsH. Cheng and Echols (1987) reported the results of two-dimensional gel electrophoresis showing that some 13 proteins were stabilized in the *hflA* mutant. These proteins could be the CII-class substrates of FtsH. Although these authors did not discuss this way, their results also indicate that some proteins, especially those at the basic region of the gel, decreased or disappeared in the mutant cells. The latter proteins could be the SecY-class of the FtsH substrates.

The HflKC requirement of the YccA11-mediated inhibition of proteolysis can be explained in different ways. For instance, YccA11 may effectively be brought into the FtsH system by the action of HflKC. Alternatively, it somehow activates the inhibitory action of HflKC. A more intersting but speculative mechanism may be that HflKC is a factor negatively controlling the hypothetical dislocation process of a membrane protein substrate, thus keeping the substrate (YccA11 in this case) bound to the membrane sector of FtsH. There are two possibilities for the mode of the HflKC action. First, it may exert transmembrane control over the cytoplasmic protease activity of FtsH. Second, it may directly associate with a membrane protein substrate to retard its presentation (dislocation?) to the protease domain. These possibilities are not mutually exclusive.

Although details of the molecular mechanisms remain to be clarified, the present study revealed that there are at least two modes of entry into the FtsH proteolytic system, and only that for membrane protein substrates is interfered with by the mutant form of YccA. Observations obtained using the YccA11 mutant protein and its derivative with an cytoplasmic extension provided intersting speculations and,

hence, interesting future experimental approaches to the processes required for the ATP-dependent proteolysis of integral membrane proteins in *E. coli*.

§ 4 Materials and methods

1. Bacterial strains and plasmids

E. coli strains and plasmids used in this study are shown in Table III and Table IV, respectively.

Table III Strains used in this study

Strain	Genotype	Source or Reference
MC4100	*Δlac araD thiA rpsL relA*	Casadaban,1976
BL21	F⁻ *ompT* [*lon*] *hsdS*$_B$ ($r_B^- m_B^-$)	Grodberg and Dunn, 1988
AD16	*Δpro-lac thi* /F' *lacI*^{q} Z^{M15} Y$^+$ *pro*$^+$	Y. Akiyama[a]
AD179	MC4100, *ΔompT*	Akiyama and Ito, 1990
AD202	MC4100, *ompT::kan*	Akiyama and Ito, 1990
AD233	MC4100, *zgj-231*::Tn*10 ftsH101*	Y. Akiyama, unpublished
JP5042	*cycA purA rif-356 str-724*	Russell, 1972
IQ85	MC4100, *zhd-33*::Tn*10 secY24*	Shiba *et al.*, 1984
AR796	MC4100, *zhd-33*::Tn*10 zhj-3198*::Tn*10* Kan	T. Ogura[a]
AR797	MC4100, *zhd-33*::Tn*10 zgj-3198*::Tn*10*Kan *ftsH1*	T. Ogura[a]
AK315	AD16, *zgj-231*::Tn*10 ftsH101*	this study[a]
AK318	AD16, *zgj-231*::Tn*10*	this study[a]
AK342	MC4100, *secY24 zgj-231*::Tn*10*	this study[a]
AK345	MC4100, *secY24 zgj-231*::Tn*10 ftsH101*	this study[a]
AK406	AD16, *secY24 zhd-33*::Tn*10*	this study[a]
AK420	IQ85, *zgj-3198*::Tn*10*Kan	this study[a]
AK421	IQ85, *zgj-3198*::Tn*10*Kan *ftsH1*	this study[a]
AK519	AD16, *zgj-460*::Tn*5*	this study[a]
AK520	AD16, *zgj-460*::Tn*5 zgj-520*::IS*1*A	this study[a]
AK521	AD16, *zgj-460*::Tn*5 ftsJ101*::IS*10*L	this study[a]
AK523	AD16, *zgj-460*::Tn*5 zgj-523*::IS*10*R	this study[a]
AK524	AD16, *zgj-460*::Tn*5 zgj-524*::IS*1*A	this study[a]
AK525	AD16, *zgj-460*::Tn*5 zgj-525*::IS*1*A	this study[a]
AK526	AD16, *zgj-460*::Tn*5 ftsJ100*::IS*1*A	this study[a]
AK646	AD16, *zgj-460*::Tn*5 ftsH102*	this study[a]

AK861	AD16, *zjf-803*::Tn*5*	this study[b]
AK863	AD16, *zjf-803*::Tn*5 hflC9*	this study[b]
AK865	AD16, *zjf-803*::Tn*5 hflK13*	this study[b]
AK897	AD16, *zcc-554*::Tn*5*	this study[d]
AK904	AD16, *yccA11 zcc-554*::Tn*5*	this study[d]
AK990	AD16, *ΔhflK-hflC*::*kan*	this study[b]
AK1127	AD16, *hflC*::*tet*	this study[c]
AK1129	AD16, *ΔhflK-hflC*::*tet*	this study[b]
AK1181	AD202, *ΔftsH*::(*ftsH*$^+$-*his*$_6$-*myc*, *tet*)	this study[b]
AK1191	AD16, *secY24 rpsE zhd-33*::Tn*10*	this study[b]
AK1194	AD16, *secY24 rpsE ΔhflK-hflC*::*tet*	this study[b]
AK1244	AK1194, *zgj-460*::Tn*5 zgj-525*::IS*1*A	this study[b]
AK1272	AK1441, F' *lacI*^{q} / *ΔftsH*::(*ftsH*$^+$-*his*$_6$-*myc*, *tet*)	this study[b]
AK1301	AD16, *ΔhflK-hflC*::*tet zgj-460*::Tn*5 zgj-525*::IS*1*A	this study[c]
AK1339	AD16, *zjf-803*::Tn*5 hflA150*	this study[c]
AK1396	AD16, *yccA11 zcc-554*::Tn*5 ΔhflK-hflC*::*tet*	this study[d]
AK1405	AD179, *zcc-554*::Tn*5 yccA11*	this study[d]
AK1441	AD179, *ΔhflK-hflC*::*kan*	this study[d]
AK1485	AD179, *yccA*::*kan*	this study[d]
AK1653	AD16, *zjf-803*::Tn*5 ilv*::Tn*10* Δ(*atpB-atpC*)	this study[d]
AK1654	AD16, *zjf-803*::Tn*5 hflC9 ilv*::Tn*10* Δ(*atpB-atpC*)	this study[d]
AK1655	AD16, *zjf-803*::Tn*5 hflK13 ilv*::Tn*10* Δ(*atpB-atpC*)	this study[d]
AK1656	AD16, *zcc-554*::Tn*5 yccA11 ilv*::Tn*10* Δ(*atpB-atpC*)	this study[d]
AK1748	AD179, *yccA11 zcc-554*::Tn*5 ΔhflK-hflC*::*tet*	this study[d]

[a-d] Details of the constructions were described elsewhere: a, Kihara *et al.* (1995); b, Kihara *et al.* (1996); c, Kihara *et al.* (1997); d, Kihara *et al.*, submitted.

Table IV Plasmids used in this study

Plasmid	Cloned Genes	Marker	Replicon or vector	Source or reference
pMW118	vector	Amp	pSC101	Nippon Gene
pMW119	vector	Amp	pSC101	Nippon Gene
pHSG575	vector	Cm	pSC101	Takeshita *et al.* (1987)
pKY225	vector	Amp	pBR322	Taura *et al.* (1993)
pKY238	vector	Cm	pACYC184	Shimoike et al. (1992)
pBAD18	vector	Amp	pBR322	Guzman *et al.* (1995)
pTWV228	vector	Amp	pBR322	Takara Shuzo Co.
pTWV229	vector	Amp	pBR322	Takara Shuzo Co.

pBlueScript SK$^-$	vector	Amp	pUC	Stratagene
pKK223-3	vector	Amp	pBR322	Brosius and Holy (1984)
pKY248	p*lac-secY*	Cm	pKY238	Taura *et al.* (1993)
pKY258	p*lac-secY-lacZα*	Cm	pKY238	Baba *et al.* (1994)
pKY318	p*lac-secY*	Amp	pKY225	Taura *et al.* (1993)
pSTD181	p*lac-atpB*	Cm	pHSG575	Akiyama *et al.* (1996b)
pSTD113	p*lac-ftsH-his$_6$-myc*	Amp	pBlueScript SK$^-$	Akiyama *et al.* (1995)
pSTD401	p*lac-ftsH*	Cm	pHSG575	Akiyama *et al.* (1994a)
ptaccII$_{Y42}$	p*tac-cII*	Amp	pKK223-3	C. Herman[c]
pKH132	*divE-yccA*	Amp	pMW119	this study[d]
pKH135	*yccA*	Amp	pMW119	this study[d]
pKH142	*hflX-hflK-hflC*	Amp	pMW119	this study[b]
pKH144	*hflX*	Amp	pMW119	this study[b]
pKH145	p*lac-hflK*	Amp	pMW119	this study[b]
pKH146	p*lac-hflC*	Amp	pMW119	this study[b]
pKH169	*hflX-hflK-hflC*	Amp	pTWV228	this study[b]
pKH178	p*ara-hflK-hflC*	Amp	pBAD18	this study[b]
pKH182	*hflX-ΔhflK-hflC::kan*	Amp	pTWV228	this study[b]
pKH189	*yccA11*	Amp	pMW119	this study[d]
pKH191	p*lac-hflK-hflC*	Amp	pMW119	this study[b]
pKH193	p*lac-hflK-hflC9*	Amp	pMW119	this study[b]
pKH194	p*lac-hflK13-hflC*	Amp	pMW119	this study[b]
pKH198	p*lac-ftsH*	Amp	pMW118	this study[b]
pKH201	*hflX-ΔhflK-hflC::tet*	Amp	pTWV228	this study[b]
pKH274	p*ara-cII*	Amp	pBAD18	this study[c]
pKH290	p*lac-hflK13-hflC9*	Amp	pMW119	this study
pKH303	p*lac-yccA-his$_6$-myc*	Amp	pUC119	this study[d]
pKH330	p*lac-yccA-his$_6$-myc*	Amp	pTWV229	this study[d]
pKH331	p*lac-yccA11-his$_6$-myc*	Amp	pTWV229	this study[d]
pKH356	p*lac-N8-yccA11-his$_6$-myc*	Amp	pTWV229	this study[d]

[b-d] Details of thier constructions were described elsewhere (see notes for Table III).

2. Media

L medium (Davis *et al* ., 1980), peptone medium (Ito *et al.*, 1983), M9 medium (Silhavy *et al.*, 1984), and minimal E medium (Shiba *et al.*, 1986) were used. Amp (50 μg/ml) and/or Cm (20 μg/ml) were included for growing plasmid-bearing strains. Tetracycline (8 or 25 μg/ml), kanamycin (12.5 or 25 μg/ml), and spectinomycin (50

μg/ml) was added to L agar as required for selecting transductants.

3. Isolation of mutants defective in degradation of excess SecY

Cells of AK406 (*secY24*) were plated on minimal E agar at 42°C, a nonpermissive temperature for the *secY24* mutants (Shiba *et al.*, 1986). Temperature-resistant colonies that appeared at frequencies of 10^{-7}-10^{-6} were pooled and transformed with pKY258 (*secY-lacZα*). Transformants were selected at 37°C on L agar containing 40 μg of XG per ml, 0.25 mM tPEG, and 1mM IPTG, and those with blue colony color (which appeared at frequencies of 5-10%) were saved. Among them about 40% showed cold-sensitivity for growth, and we initially used these cold-sensitive mutants for further analysis. Their responsible mutations (see Fig. 9) were all found to be linked with *zgj-460*::Tn*5* (see below). They were introduced into AD16 by joint transduction with the transposon. Strains thus constructed were AK646 (AD16, *zgj-460*::Tn*5 ftsH102*), AK526 (*ftsJ100*::IS*1*A), AK521 (*ftsJ101*::IS*10*L), AK520 (*zgj-520*::IS*1*A), AK524 (*zgj-524*::IS*1*A), AK525 (*zgj-525*::IS*1*A), and AK523 (*zgj-523*::IS*10*R). The insertion *zgj-460*::Tn*5* linked to *ftsH* (85% cotransduction) was selected from random transpositions by joint P1 transduction with the *argG*$^+$ marker (Kleckner *et al.*,1978).

In a different series of mutant isolation, we repeated the screening as described above expect that we did not look for cold-sensitive colonies. We obtained 20 mutants from 10 independent bacterial cultures. P1 transduction mapping using *zgj-460*::Tn*5* showed that 15 of them were probably *ftsH* mutants. A transposon insertion, named *zjf-803*::Tn*5*, that is linked (at 85% co-transduction) with one of the mutation (named *hflK13*) was isolated by random transposition of Tn*5* followed by P1 joint transduction with the mutation. Transduction mapping showed that two of the remaining four mutants also have mutations in the *hflA* region. Using the *zjf-803*::Tn*5* marker, mutations were introduced into AD16. Strains thus constructed were AK863 (AD16, *zjf-803*::Tn*5 hflC9*) and AK865 (AD16, *zjf-803*::Tn*5 hflK13*). AK861 was the *hflK*$^+$-*hflC*$^+$ counterpart of the above strains. We isolated another Tn*5* insertion, named *zcc-*

554::Tn*5*, which is linked (at 90% co-transduction) with one of the remaining two mutations (*yccA11*), after random transposition of Tn*5* followed by P1 cotransduction with the mutation. The remaining last mutant proved to be a sibling of the *yccA11* mutant, and we used the *yccA11* mutant for further characterization. Using *zcc-554*::Tn*5* as a selective marker, we constructed AK904 (AD16, *yccA11 zcc-554*::Tn*5*) and AK897 (AD16, *zcc-554*::Tn*5*) by P1 transduction.

4. Nucleotide Sequence Determination of the *ftsH* Region

The *ftsH* coding region was amplified from the chromosome by PCR, using upstream primer (5'-GAATTCCACAGTTGTAATAAGAGG-3') and downstream primer (5'-GCTCTAGATACAGTCATCTGATGCGG-3'), with attached recognition sequences of *Eco*RI and *Xba*I, respectively. Amplified fragments were digested with these enzymes and cloned into pTWV229 for single strand preparation and determination of nucleotide sequences by the chain termination method (Sanger *et al.*, 1977), using the 7-deaza Sequenase kit (USB). The universal primer (5'-GTTTTCCCAGTCACGACGTTGTA-3') as well as a series of synthetic primers (5'-GGATCCGAAATTACTGGA-3', 5'-GGTCGGTCCTCCGGGTAC-3', 5'-CCTGCTGCG TCCTGGCC-3', 5'-CCTGGTGCCGGAACACGA-3', 5'-GAAACTGCACGTATCAT-3') which corresponded to regions upstream or within the *ftsH* gene were used. Any deviations from the wild-type sequence were confirmed by sequencing at least 3 independent clones. For amplification of the upstream region of *ftsH*, following primers were designed: 5'-CGGACTCTTCTCGTGCAC-3' and 5'-TCGCCAGCAGGTT TTTACCGGT-3'. The amplified fragments were treated with T4 DNA polymerase and *Kpn*I, and ligated with *Kpn*I-*Hinc*II digested pTWV228. The universal primer was used for sequencing.

5. Cloning and sequencing of the mutated *hflK* and *hflC* genes

For cloning the wild-type and mutant forms of *hflK* and *hflC* genes, chromosomal

DNA prepared from AK861 (*hflK*$^+$ *hflC*$^+$), AK863 (*hflC9*), or AK865 (*hflK13*), respectively, was partially digested with *Sau*3AI and ligated into the *Bam*HI site of pMW119. Transformants that were *purA*$^+$ and Amp^R were selected using strain JP5042 (*purA*$^-$). Thus, plasmids containing the *hflA-purA* interval were obtained. They were pKH160 carrying a 9.0 kb *hflK*$^+$-*hflC*$^+$-*purA* insert, pKH162 carrying a 11 kb *hflK*$^+$-*hflC9-purA* insert and pKH163 carrying a 14 kb *hflK13-hflC*$^+$-*purA* insert. The 2.1 kb *Dra*I-*Sal*I fragment of pKH162 (*hflC9*) or the 1.3 kb *Hpa*I-*Eco*RI fragment of pKH163 (*hflK13*) was cloned into M13 mp18 or M13 mp19, respectively, from which single-stranded DNAs were prepared for determination of nucleotide sequences as described above. The universal primer as well as a series of synthetic primers (5'-CAAGTTCGTATGC CGATCG-3', 5'-GCAACTCTGAGGGAAATGG-3', 5'-GCGTGGTAACACGCTTTGG-3', 5'-GTCAGGCTACCGACAGCGC-3', 5'-CATTCGTGAAGCAGAAGCG-3', 5'-AAA GGTGGCAACCTGATGG-3', 5'-GCGTAAGTCAGTTATCGCG-3', 5'-AAGAAAGAC CTGATCGTCG-3', 5'-AAGATGAAGTTACTACCCC-3', 5'-AGAAGCGGAAAAACT GCGC-3') that corresponded to the regions upstream or within the *hflK-hflC* genes were used.

6. Cloning and sequencing of the *yccA*$^+$ and *yccA11* nucleotide sequences

To clone *yccA11*, chromosomal DNA from AK904 was first digested with *Eco*RI, and about 1.3 kb fragments were isolated and digested further with *Taq*I. About 0.9 kb fragments were then cloned into the *Acc*I site of pMW119. One of the resulting plasmids, pKH189, proved to carry the *yccA11* marker, since it dominantly stabilized the SecY-LacZα protein. A 0.7 kb *Vsp*I fragment from pKH135 (*yccA*$^+$) as well as that from pKH189 (*yccA11*) were cloned into M13 mp18, from which single stranded DNAs were prepared for the determination of the nucleotide sequences as described above. The universal primer as well as synthetic primers (5'-TCCGGGTCTGATTCT GACG-3', 5'-AGATATGTCGTTCCTCGGC-3') were used. The nucleotide sequence of *yccA*$^+$ was identical to that previously reported (accession number, X00547; Tamura

et al., 1984).

7. Antibodies

For immunoblotting and immunoprecipitaion of SecY, antisera against N-terminal and C-terminal sequences of SecY (Shimoike *et al.*, 1995) were used. Anti-OmpA serum was provided by Y. Anraku. Anti-β-lactamase was purchased from 5 Prime 3 Prime, Inc.. For detection of c-Myc epitope tag, Ab-1 (Oncogene Science, Inc.) or A-14 (Santa Cruz Biotechnology, Inc.) was used. Anti-MBP (Shimoike *et al.*, 1995), Anti-GroEL (Akiyama *et al.*, 1994a), and anti-F_0 *a* (Futai *et al.*, 1989) were described previously. Anti-FtsH provided by W. Wickner (Dartmouth Medical School) was used for experiments shown in Fig. 10 and Fig. 16. For other experiments, we prepared anti-FtsH serum using a synthetic peptide (NH_2-TNRPDVLDPALLRPGRC-COOH) that corresponded to amino acids 297 to 312 of FtsH, and conjugated with Keyhole Limpet Hemocyanin. Anti-HflKC serum used in Fig. 16-19 were provided by the laboratory of the late Dr. H. Echols (University of California, Berkeley). We also prepared antisera against the purified HflKC complex and against the HflK subunit in rabbits by standard procedures.

8. Pulse-chase and immunoprecipitation under denaturing conditions

Typical procedure for pulse-chase and immunoprecipitation experiments is as follows. Cells were grown to an early log phase in M9 medium supplemented with 18 amino acids (20 μg/ml, other than Met and Cys), thiamine (2 μg/ml), 0.4% carbon sources (glucose or glycerol), and appropriate antibiotics. Plasmid-encoded genes were induced with 1 mM IPTG (for the gene under the control of *lac* promoter) or with 0.4% arabinose (for the gene under control of *ara* promoter) for 10 min, and cells were pulse-labeled with [^{35}S]methionine followed by chase with 200 μg/ml unlabeled L-methionine. At each time point, a portion of the labeled culture was

mixed with an equal volume of 10% TCA. After the preparations were kept on ice for 20 min or longer, protein precipitates were collected by centrifugation at 4°C, washed with acetone, and then solubilized in buffer containing 1% SDS, 50 mM TrisHCl (pH 8.1), and 1 mM EDTA. Samples are diluted with 33 fold excess Lubrol buffer (50 mM TrisHCl (pH 8.1), 0.1 mM EDTA, 0.15 M NaCl, and 0.1% Lubrol) or Triton Buffer (50 mM TrisHCl (pH 8.1), 0.1 mM EDTA, 0.15 M NaCl, and 2% Triton-X100), and insoluble materials are removed by centrifugation. Then, they are incubated with appropriate antibodies together with proteinA-Sepharose beads at 4°C for 12-16 hr. Immunocomplexes were isolated by centrifugation and washed twice with the same buffer and once with 10 mM TrisHCl (pH 8.1). Proteins were separated by SDS-PAGE and visualized by autoradiography using a Fujix bioimaging analyzer BAS2000 (Fuji Film).

9. Examination of *in vivo* stability of CII

Stability of cloned and overproduced CII was examined by growing cells harboring pKH274 (*para-cII*) to a log phase in M9 medium supplemented with 18 amino acids (20 μg/ml, other than Met and Cys), thiamine (2 μg/ml), 0.4% glycerol and 50 μg/ml Amp, inducing CII with 0.4% arabinose for 10 min, and pulse-labeling the cells with [^{35}S]methionine for 0.5 min and chasing with unlabeled L-methionine (200 μg/ml) for indicated periods. SDS-PAGE (15% gel, Laemmli, 1970) of total cell proteins, directly precipitated with 10% TCA, gave a well separated band of CII. Stability of CII in λ-infected cells was determined by pulse-chase experiments as described above, using UV-irradiated (400 W/m^2 for 10 min at 254 nm) and λ$^+$-infected cells (multiplicity of infection, about 5) as described (Gottesman *et al.*, 1981). The band of CII under the latter conditions was minor but distinct. In both cases, intensities of CII were quantitated by Fujix BAS2000 bioimaging analyzer.

10. Immunoblotting

Proteins were separated by SDS-PAGE and electroblotted onto an Immobilon PVDF (Millipore) or a Zeta-Probe membrane filter (Bio-Rad). Filters were blocked with 5% skim milk dissolved in phosphate-buffered saline at 42°C for 1 hr or at 4°C overnight and incubated in the same buffer with appropriately diluted antiserum at room temperature for 1hr. They were then washed and treated with goat anti-rabbit immunogloblin G conjugated with horseradish peroxidase (Bio-Rad); the reaction was visualized on X-ray film by using an ECL Western blot detection kit (Amersham).

11. Crosslinking using DSP

Cells were grown to an early-log phase in M9 medium supplemented with 18 amino acids (20 μg/ml, other than Met and Cys), thiamine (2 μg/ml) and 0.4% glucose, and were pulse-labeled for 10 min with [^{35}S]methionine. The culture was chilled and cells were harvested, washed with buffer I (50 mM HEPES/KOH (pH 8.0), 50 mM KCl, 1 mM DTT, 20% glycerol), and resuspended in the same buffer. After disruption of cells by sonication and removal of cell debris by centrifugation (4000 x g for 10 min), total membrane fraction was precipitated by centrifugation (500,000 x g for 45 min). Membranes were then suspended in buffer I without DTT and treated with DSP (1/40 volume of 30 mg/ml solution in DMSO to give a final concentration of 0.75 mg/ml) at 4°C for 16 hr. Mock treatment samples received DMSO. The reaction was terminated by addition of 0.2 M ammonium acetate followed by incubation at 4°C for 10 min. Membrane proteins were then solubilized with 1% SDS by incubating at 37°C for 5 min. FtsH and HflKC proteins were immunoprecipitated after dilution of the SDS-solubilized samples with Lubrol buffer. Antigen-antibody complexes were isolated with protein A-Sepharose, and solubilized with SDS sample buffer with 10% 2-mercaptoethanol. Thus crosslinkings were cleaved before separation by subsequent SDS-PAGE and visualization of proteins by autoradiography.

12. Purification of HflKC

Cells of BL21/pKH178 were grown to a mid-log phase at 37°C in 16 liter of M9 medium supplemented with amino acids (20 μg/ml), thiamine (2 μg/ml), 0.4% glycerol, 0.4% maltose, and 50 μg/ml Amp. The synthesis of the HflKC protein from the plasmid was induced by 0.2% arabinose 3 hr before harvesting the cells. Cells were washed with 10 mM Tris-HCl (pH 7.2), suspended in 180 ml of buffer II (50 mM Tris-HCl (pH 7.2), 20% glycerol, 1 mM EDTA, 1 mM DTT), and disrupted by repeated (2 times) passages through a French pressure cell at 7500 p. s. i.. After centrifugation at 38,000 rpm for 1 hr in a Beckman type 70 Ti rotor, membrane pellets were resuspended in buffer II and solubilized by adjusting the buffer composition to buffer III (0.5% NP40, 50 mM Tris-HCl (pH 7.2), 10% glycerol, 1 mM DTT) and incubating at 0°C for 1 hr. Samples were centrifuged again and the supernatant was applied to a DEAE-Sepharose fast flow column (90 ml bed volume) that had been equilibrated with buffer IV (50 mM Tris-HCl (pH 7.2), 10% glycerol, 1 mM DTT, 0.1% NP40). The column was then developed with 5 x column volume of 0-300 mM KCl gradient in buffer IV. HflKC enriched fractions were diluted two fold with buffer IV, adsorbed to a small (8 ml) column of DEAE-Sepharose fast flow and concentrated by a step elution with 300 mM KCl in buffer IV. The sample was applied to Superdex 200 gel filtration column (2.6 x 60 cm; Pharmacia LKB) connected to a Waters 650E HPLC and pre-equilibrated with buffer V (buffer IV plus 150 mM KCl). Fractions with the highest purity of HflKC were pooled and applied to an Econo-Pac hydroxyapatite column (5 ml, from Bio Rad) connected to a Waters 650E HPLC. The column was pre-equilibrated with buffer VI (50 mM Tris-HCl (pH 7.2), 20% glycerol, 1 mM DTT, 0.1% NP40, 50 mM KCl, 10 mM potassium phosphate (pH 7.2)), washed with 1 column volume of buffer VII (buffer VI without KCl), and developed with 4 column volume of 0-1 M KCl gradient in buffer VII. We found that about a half of HflKC was eluted with high concentration of KCl, while the other half required further elution by phosphate buffer. The KCl eluate was used in

this work.

13. Purification of FtsH-His_6-Myc

Cells of AK1272 carrying pSTD113 were grown at 37°C to a mid-log phase in 8 liter of M9 medium supplemented with 0.2% casamino acids, thiamine (2 μg/ml), 0.4% glycerol, and 50 μg/ml Amp. IPTG (final concentration 1 mM) was added 3 hr before collection of cells by centrifugation (9000 g for 10 min at 4°C). Cells were washed with 10 mM Tris-HCl (pH 7.2), suspended in buffer A (50 mM Tris-HCl (pH 7.2), 20% glycerol, 100 mM KCl, 5 mM $MgCl_2$, 1 mM Pefabloc (Boehringer Manheim), 10 mM 2-mercaptoethanol), and disrupted as described in the previous section. Membranes were prepared as described in the previous section, resuspended in buffer A, and solubilized by adjusting the buffer composition to buffer B (0.5 % NP40, 20 mM Tris-HCl (pH 7.2), 10 % glycerol, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 1 mM ATP, 20 mM imidazole, 1 mM Pefabloc, 0.5 M KCl) and incubating at 0°C for 1 hr. After centrifugation (38,000 rpm in a 70 Ti rotor, 1 hr), supernatant was loaded onto a Ni-NTA-agarose column (10 ml) equilibrated with buffer B. The column was washed with 20 ml of buffer C (same as buffer B except that NP40 was 0.1 % and KCl was 1 M) and with 20 ml of buffer D (same as buffer C except that KCl was 50 mM). Bound proteins were eluted with a 20 to 250 mM gradient of imidazole in buffer D. Fractions enriched in FtsH-His_6-Myc were pooled, and concentrated by a step elution from a small DEAE-Sepharose fast flow column (1 ml) with buffer E (50 mM Tris-HCl (pH 7.2), 300 mM KCl, 0.1% NP40, 10% glycerol, 5 mM $MgCl_2$, 1 mM ATP, 1 mM DTT). The sample was then fractionated by a Superose 6 gel filtration column equilibrated with buffer F (same as buffer E except that KCl was 150 mM). Fractions containing FtsH-His_6-Myc were pooled and stored at –80°C.

14. Isolation of a complex containing FtsH-His_6-Myc using Ni-NTA agarose

Cells of AD202 and AK1181 were grown to a mid-log phase in P medium. Cells

were treated with 40 μM CCCP for 5 min, washed with 10 mM Tris-HCl (pH 8.1), and suspended in buffer G (10 mM Tris-HCl (pH 8.1), 50 mM KCl, 20 % glycerol, 10 mM 2-mercaptoethanol). In our initial experiments we added CCCP to deplete ATP, which was suspected to induce artifical degradation of FtsH-His_6-Myc. We later found that ATP stimulated the FtsH-HflKC binding within the membrane, but did not induce degradation of FtsH-His_6-Myc. In any case, essentially similar results were obtained without CCCP treatment. Total membranes were prepared as described in the previous sections and solubilized in buffer H (50 mM Tris-HCl (pH 8.1), 300 mM KCl, 3 mM imidazole, 10% glycerol, 0.5% NP40, 10 mM 2-mercaptoethanol) at 0°C for 1 hr. After removal of insoluble materials by centrifugation, proteins were incubated with Ni-NTA agarose, washed 3 times with buffer H, and eluted with buffer J (buffer H except that imidazole was 250 mM). Protein eluates were precipitated by 5% TCA, solubilized with 1% SDS-50 mM Tris-HCl (pH 8.1)-1 mM EDTA, separated by SDS-PAGE, and subjected to immunoblotting with anti-FtsH and anti-HflKC.

15. Purification of YccA-His_6-Myc

Cells of AD202/pKH303 were grown at 37°C to an early-log phase in 3 liters of L medium supplemented with 0.2% glucose and 50 μg/ml Amp. Cyclic AMP (final concentration, 1 mM) was added 4 hr before collection of cells by centrifugation (9000 x g for 10 min at 4°C). Cells were washed with 50 mM Tris-HCl (pH 8.1), suspended in buffer K (20 mM Tris-HCl (pH 8.1), 20% glycerol, 100 mM KCl, 10 mM 2-mercaptoethanol, 1 mM Pefabloc (Boehringer Manheim)), and incubated with EDTA (final concentration, 2 mM) and lysozyme (final concentration, 200 μg/ml) at 0°C for 30 min. Membranes were prepared as described above, resuspended in buffer K, solubilized by adjusting the buffer composition to that of buffer L (0.5% NP40, 20 mM Tris-HCl (pH 8.1), 10% glycerol, 300 mM KCl, 10 mM 2-mercaptoethanol, 1 mM Pefabloc) and incubating at 0°C for 1 hr. After centrifugation (38000 r.p.m. in a 70 Ti rotor, 1 hr), supernatant was loaded onto Ni-NTA-agarose column (5 ml) equilibrated

with buffer L. The column was washed with 10 ml of buffer M (0.1% NP40, 20 mM Tris-HCl (pH 8.1), 10% glycerol, 50 mM KCl, 10 mM 2-mercaptoethanol). The column was washed with 15 ml of buffer N (buffer M with 30 mM imidazole), and bound proteins were eluted with 15 ml of buffer P (buffer M containing 250 mM imidazole).

16. HflKC and YccA-His_6-Myc binding in digitonin solution

Purified YccA-His_6-Myc preparation described above was subjected to buffer exchange using NAP-10 column (Pharmacia Biotech) equilibrated with 20 mM Tris-HCl (pH 8.1), 10% glycerol, 100 mM KCl, 0.1% NP40, 10 mM 2-mercaptoethanol. 1.3 μg YccA-His_6-Myc in 5 μl was mixed with either purified HflKC (1.3 μg in 20 μl) or buffer Q (10 mM Tris-HCl (pH 8.1), 10% glycerol, 5 mM $MgCl_2$, 0.1% NP40, 10 mM 2-mercaptoethanol). The mixture was diluted with 6 volumes of buffer Q' (50 mM Tris-HCl (pH 8.1), 1% digitonin, 200 mM KCl, 10% glycerol, 10 mM 2-mercaptoethanol) followed by incubation at 0°C for 1 hr. The samples were then incubated with Ni-NTA-agarose, washed three times with buffer Q', and eluted with buffer R (buffer Q' containing 250 mM imidazole).

17. Immunoprecipitaion of the FtsH complex using anti-FtsH antibodies

Cells of AD202 were grown to an early-log phase in M9 medium supplemented with 18 amino acids (20 μg/ml, other than Met and Cys), thiamine (2 μg/ml), and 0.4% glucose, and pulse-labeled for 10 min with [^{35}S]methionine. After treatment with 40 μM CCCP as described above, cells were collected by centrifugation, and suspended in buffer S (50 mM Tris-HCl (pH 8.1), 50 mM KCl, 20% glycerol, 1 mM DTT, 5 mM EDTA) for preparation of total membranes as described above. Membrane proteins were solubilized in buffer T (50 mM Tris-HCl (pH 8.1), 300 mM KCl, 10% glycerol, 0.5% NP40, 1 mM DTT) at 0°C for 1 hr. After removal of insoluble materials by centrifugation, proteins were incubated with affinity-purified anti-FtsH IgG together with protein A-Sepharose beads at 4°C for 4 h. Immunocomplexes were

isolated by centrifugation and washed twice with buffer T and once with buffer U (buffer T except that NP40 was 0.05%). Proteins were separated by SDS-PAGE and visualized by autoradiography using a Fujix bioimaging analyzer BAS2000 (Fuji Film). To address the specificity of the precipitated proteins, antibodies were pretreated with excess antigen peptides (5 mg/ml) used for immunization.

18. Immunoprecipitaion of the FtsH/HflKC complex using anti-HflKC antibodies

Cells were grown to an early log phase in M9 medium supplemented with 18 amino acids (20 μg/ml, other than Met and Cys), thiamine (2 μg/ml) and 0.4% glucose, and labeled for 30 min with [^{35}S]methionine. Cultures were chilled and mixed with 0.02% sodium azide and 0.01% Cm. Cells were collected by centrifugation, suspended in buffer W (50 mM Tris-HCl (pH 8.1), 20% glycerol, 100 mM KCl, 1 mM DTT), and incubated with EDTA (final concentration, 2 mM) and lysozyme (final concentration, 200 μg/ml) at 0°C for 30 min followed by disruption by sonication. After removal of cell debris by centrifugation (4000 x g for 10 min), the total membrane fraction was isolated by centrifugation (500,000 x g for 45 min). Membranes were then suspended in buffer A and solubilized by addition of 40 fold excess buffer Y (50 mM Tris-HCl (pH 8.1), 1% digitonin, 200 mM KCl, 10% glycerol, 1 mM DTT). After removal of insoluble materials by centrifugation, proteins were incubated with anti-HflKC serum together with protein A-Sepharose beads at 4°C for 12 hr. The anti-HflKC serum had been preincubated with non-labeled solubilized membrane fraction prepared from AK1441 (Δ*hflK-hflC*) to reduce the background. Immunoprecipitates were isolated by centrifugation, washed three times with buffer Y, and suspended in SDS Sample buffer. Proteins were separated by SDS-PAGE and visualized by autoradiography using a Fujix bioimaging analyzer BAS2000 (Fuji Film). To address the specificity of the precipitated proteins, control reactions were included, in which antibodies had been pre-treated with excess purified HflKC.

19. Preparation of radio-labeled CII protein

Cells of AK1301/ptac-cII$_{Y42}$ were grown at 37°C to an early-log phase in 20 ml of M9 medium supplemented with 18 amino acids (20 μg/ml each other than Met and Cys), thiamine (2 μg/ml), 0.4% glycerol, and 50 μg/ml Amp. The synthesis of CII was induced with 1 mM IPTG for 10 min and cells were labeled with 3.7 MBq of [^{35}S]methionine (>29.6 TBq/mmol) for 1 min. The culture was then chilled and mixed with 200 μl of 2% NaN_3 and 800 μl of 2.5 mg/ml Cm. Cells were collected by centrifugation and suspended in 640 μl of buffer Z (50 mM TrisHCl (pH 8.1) containing 2 mM EDTA, 5% glycerol, and 1 mM dithiothreitol (DTT)) supplemented with 1 mM Pefabloc, and incubated on ice with lysozyme (200 μg/ml) for 30 min. Cells were disrupted by sonication and aggregates of the CII protein were collected by centrifugation at 5 x 10^5 g for 45 min and dissolved in 400 μl buffer Z supplemented with 0.05% deoxycholate, 1 M NaCl, and 0.1 M $MgCl_2$ with incubation at 4°C for 1 hr (Cheng *et al.*, 1988; Ho *et al.*, 1982). After centrifugation (as above), supernatant was subjected to buffer exchange using NAP-10 column equilibrated with 50 mM TrisHCl (pH 7.2) containing 10% glycerol, 5 mM $MgCl_2$, 30 mM KCl and 1 mM DTT. This preparation gave a single radioactive band (apparent molecular mass, 11 kDa) upon SDS-PAGE. Identity of this protein with CII was shown by its *tac*-promoter specific and plasmid-specific appearance.

20. *In vitro* degradation assay using FtsH-His$_6$-Myc

100 ng of a purified SecY preparation (Akimaru *et al.*, 1991) was incubated with FtsH-His$_6$-Myc (300 ng) in a standard reaction mixture containing 50 mM Tris-HCl (pH 7.2), 10% glycerol, 0.1% NP40, 5 mM $MgCl_2$, 1 mM DTT, 5 mM ATP, and 25 mM zinc acetate. HflKC (1300 ng) was included as required. The mixture was incubated at 37°C, during which portions were withdrawn and mixed with 2 x SDS sample buffer (125 mM Tris-HCl (pH 6.8), 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, a trace amount of Bromophenol blue). The SecY protein was detected by immunoblotting

after SDS-PAGE.

The radioactive CII preparation (1-1.5 x 10^4 cpm) was incubated with FtsH-His_6-Myc in a standard reaction mixture containing 50 mM TrisHCl (pH 7.2), 10% glycerol, 0.1% NP40, 5 mM $MgCl_2$, 5 mM ATP, 25 mM KCl, 1 mM DTT, 25 μM zinc acetate, and 0.5 mg/ml bovine serum albumin. Preincubation of FtsH-His_6-Myc with HflKC was done as described above. The radioactivities of the CII protein, separated by SDS-PAGE, were determined using a Fujix bioimaging analyzer BAS2000 (Fuji Film).

21. Hfl phenotype tests

Lysogenization frequency of λ was measured essentially as described (Banuett *et al.*, 1986; Herman *et al.*, 1993). Briefly, cells were infected with λ^+ at a multiplicity of infection of about 0.1 and infectious centers were determined by plating with *himA* indicator cells, while lysogens were determined by plating with excess of λcI_{60} phage. The frequency of lysogenization was the ratio of lysogens to infective centers. For qualitative tests of Hfl phenotype, propagation of λc17 phage was examined as described (Banuett *et al.*, 1986; Herman *et al.*, 1993). For these tests, cells had been pre-grown in the following media: TB medium containing 0.4% maltose for cells without plasmid; TB medium containing 1 mM IPTG and 50 μg/ml Amp for cells harboring pMW119, pKH146, or pKH339; TB medium containing ampicilin with 0.4% arabinose added 1 hr before λ infection for cells harboring pBAD18 or pKH178. After the establishment of lysis-lysogeny commitment, TB-agar was used for plating all the samples.

22. Determination of the membrane orientation of HflKC

Spheroplasts and inverted plasma membrane vesicles (IMV) were prepared from AD202 as described (Yoshihisa *et al.*, 1996). They were incubated with 1 mg/ml of proteinase K at 0°C for 2 hr, followed by termination of the digestion with PMSF (final concentration, 1 mM) and precipitation of proteins with TCA. Samples were

then subjected to SDS-PAGE and immunoblotting. Anti-sera against the purified HflKC complex and against the HflK subunit were raised in rabbits by standard procedures. Anti-HflKC serum was affinity-purified.

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